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14. ABSTRACT <p>This report results from a contract tasking University of Oxford as follows: The Grantee will investigate the immunological response of subjects to one night of sleep deprivation, with respect to the following areas, (1) Are the cytokines linked with eosinophils, neutrophils, and lymphocytes, cell types which are known to be affected by sleep deprivation, changed in terms of intracellular cytokine production? (2) What is the psychoneurological link between the apparent immunodepression and cognitive impairment? (3) What is the mechanism for the noticeable decrease in plasma leptin? These results have important implications for long haul aircrews, troops deployed across time zones, and shift workers, and may lead to changes in immunization practices in order to optimize immune responses. In combination with data on individual immune phenotypes emerging from other studies, it may be possible to provide protective and supplemental measures to boost immune responses under stress.</p>					
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**Sleep Deprivation in Humans, Immunodepression and
Glutamine supplementation**

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INTRODUCTION

Sleep and Immunocompetence

Sleep has been proposed as an innate host defence, exerting effects on both specific and non-specific immunity. Increasing evidence suggests that sleep is important for proper function of the immune system (Rechtschaffen et al., 1983). Sleep deprivation can lead to immunodepression and impaired health (Benca and Quintas, 1997; Naitoh et al., 1990). Humans susceptible to the effects of sleep deprivation in a variety of different situations include: shiftworkers (Miller, 1992), intensive care patients (Schwab, 1994), infants (Kahn et al., 1994), ship crews and submariners (Miller et al., 1999; Miller et al., 2002).

In a civil aviation study, air crews flying long distance trans-meridian routes reported more health problems than short-distance personnel; these problems affected more than 30% of personnel (both pilots and cabin staff) and included colds, fatigue and sleep disturbances (Haugli et al., 1994).

Sleep disturbance is a serious problem in hospital patients. Yarrington and Mehta (1998) referred to the importance of deep sleep in promoting recovery after bone marrow transplant. They commented on the therapeutic effects of melatonin, which enhances immune response and protects against viral infections. Post-operatively, slow-wave sleep was found to be substantially decreased in patients for several days after major surgery (Orr and Stahl, 1977).

Studies of sleep-deprived animals have shown a progressive, chronic negative energy balance and gradual deterioration of health, culminating in fatal bloodstream infection without an infectious focus. In *in vitro* studies on sleep-deprived rats, Everson and Toth (2000) observed early infection of the mesenteric lymph nodes due, apparently, to bacterial translocation. The authors suggest that bacterial translocation leading to septicemia and death is the mechanism by which sleep deprivation appears to affect health adversely (but see also Rechtschaffen and Bergmann, 2001).

A few studies have investigated the effects of sleep deprivation on some aspects of immune cell function. For example, Benca et al. (1989) looked at lymphocyte proliferative ability. However, the data were contradictory. In another study, the results of administering antibiotics to rats in the first four days of sleep deprivation were inconclusive (Bergmann et al., 1996). With regard to sleep quality studies, it has been suggested that subjective sleep quality is

linked with immunodepression to an extent that is not accounted for by depression *per se* (Savard et al., 1999). They found that the amount of sleep obtained was significantly associated with the circulating numbers of CD4 and CD8 cells. White et al. (1995) observed that sleep structure distortion was a consistently replicable physiological sign of infection in HIV patients. They found that CD4 cell numbers were associated with a distortion in nocturnal sleep and suggested that this might be linked with immune function. In 1989, Brown et al. observed in rats that an 8-hr period of sleep deprivation led to the suppression of secondary antibody response. This suppression was reversed with the administration of IL-1b (a pro-inflammatory cytokine which induces sleep) or muramyl dipeptide prior to sleep deprivation. Vgontzas et al. (2001) observed increased ACTH and cortisol secretion in the evening and first half of the night in male and female insomniacs. The highest incidence of sleep disturbances correlated with the highest amount of cortisol secreted during a 4-night sleep study.

Poor sleep quality may have implications for athletes and performance (Konig et al., 2000). Boyum et al. examined the effect of continuous physical exercise, calorie deficiency and sleep deprivation on the immune system of young men participating in a military training course. It was found that non-specific phagocyte-related immunity was enhanced with increases in neutrophil and monocytes numbers from Day 1 and in the cytokine, GM-CSF. However, specific, lymphocyte-related immunity was suppressed with a reduction in CD4 T cells, CD8 T cells, B cells, NK cells and decreased serum levels of immunoglobulins during the course. Since the stress of exercise combined with sleep deprivation was observed to have an effect on GM-CSF, it would be interesting to investigate whether sleep deprivation alone in the current study will have an effect on GM-CSF levels.

However, there are comparatively few detailed studies on specific immune function parameters in humans that provide convincing evidence of a link between sleep deprivation/disorders and immunodepression (see Naitoh et al., 1990). The present investigation was designed to address those areas where there are deficiencies to provide further evidence for or against such a link, starting with acute sleep deprivation.

Glutamine and immune cells

An important fuel for some key cells of the immune system is the amino acid, glutamine. Glutamine is the most abundant amino acid in the body. It was originally classified as a non-

essential amino acid, being synthesized predominantly by skeletal muscle. However, evidence is growing that glutamine is a conditionally essential amino acid (Lacey & Wilmore, 1990). As well as providing energy, glutamine is a nitrogen donor for purine and pyrimidine nucleotide synthesis. This is essential for the synthesis of new DNA in, e.g. lymphocyte proliferation, or DNA repair and mRNA synthesis in macrophages (Ardawi and Newsholme, 1983; 1985). The concentration of plasma glutamine (p[Gln]) is decreased during stress such as major surgery or burns, or after prolonged, intensive exercise (Askenazi et al., 1980; Parry-Billings et al., 1990, 1992; Castell et al., 1996). In vitro studies have shown that, despite the presence of all other nutrients including glucose, only altering the glutamine concentration in the medium led to a reduction in lymphocyte proliferative ability. This was coupled with a decrease in response time of the cells (Parry-Billings et al., 1990).

Immune function, strenuous exercise and glutamine

The provision of glutamine, or of the branched chain amino acids (BCAA) as glutamine precursors, may help to combat transient immunodepression observed following sleep deprivation. Prolonged, exhaustive acute exercise and training can lead to a high incidence of upper respiratory tract illness (URTI) (Peters and Bateman, 1983) and to immunodepression (see Castell, 2003; see Nieman, 1994; Pedersen & Hoffman-Goetz, 2000). These exercise-induced changes can be exacerbated by factors such as exposure to hostile environments, thermal stress and sleep disturbances. Altered sleeping patterns are also a well-known problem in overtrained athletes, who suffer a high incidence of minor illnesses /infections (Budgett, 1994). Parry-Billings et al. (1992) observed a lower p[Gln] in overtrained athletes at rest compared with fit, elite athletes. Transient immunodepression occurs in endurance athletes due to the stress of undertaking prolonged, exhaustive exercise (see Nieman and Pedersen, 1999). This leads to a high incidence of illness, particularly upper respiratory tract infections (URTI). An important factor in this situation could be lower levels of glutamine in the blood, leading to lack of availability for some immune cells at a time of stress (Newsholme and Castell, 2000).

A marked decrease in self-reported illness (34%) was reported in more than 70 marathon runners receiving glutamine compared with a similar number receiving placebo (Castell et al., 1996). These individuals had a 20% decrease in p[Gln] within one hour of finishing the race. Similar observations have been made in triathletes receiving branched chain amino acid (BCAA)

supplementation (Bassitt et al. 2000). They observed a 40% decrease in the incidence of infections as well as increased lymphocyte proliferative ability and enhanced cytokine production. Bassitt et al. attributed these findings to the maintenance of p[Gln] via BCAA precursors. Bacarau et al. (2002) made similar observations. In recent studies, a decrease was observed in the incidence of infections in ninety-three marathon runners who took BCAA for four weeks prior to a race (Hiscock et al., 2001).

Leptin

The *ob* gene product leptin is released from adipose tissue. Although structurally a cytokine, it functions as a hormone in terms of energy balance. Leptin is decreased during caloric restriction: despite this, low or non-existent levels of plasma leptin have been observed in genetically obese mice and humans. The actions of leptin are mainly thought to be mediated by the hypothalamus. There is evidence that leptin can inhibit hypothalamic-pituitary-adrenal (HPA) axis function, and it has been suggested that this is linked through a direct effect inhibiting cortisol release from the adrenal gland (Bornstein et al., 1997) or via the inhibition of hypothalamic corticotrophin-releasing hormone (CRH) release (Heiman et al., 1997). Miell et al., 1996 found that glucocorticoid administration increased leptin levels in healthy humans. Cleare et al. (2001) found that patients with chronic fatigue syndrome who reported an anti-fatiguing effect of a dose of hydrocortisone also had an increase in plasma leptin concentration. Leptin also has a role in immune cell function.

Caffeine

The role of caffeine as a stimulant is well known. A questionnaire on caffeine awareness was given to the subjects in the present study. However, the effects of caffeine on immune function are rarely considered. There may be a link between caffeine and monocytes via the expression of the A2 adenosine receptor, for which caffeine is an antagonist (Huang et al., 1997). In *in vitro* studies, the inhibitory effects of IL-12 on monocytes were reversed with the addition of caffeine to the culture medium. Caffeine has been observed to have an inhibitory effect on TNF- α release by cells stimulated with *Streptococcus pneumoniae* (van Furth et al., 1995).

Cytokines

The major cytokines produced by Th2 (T-helper) cells include IL-3 and GM-CSF. Both are growth factors. The latter stimulates growth and differentiation of macrophages and granulocytes, which are important in the non-specific immune response. GM-CSF also potentiates the secretion and function of IgA and enhancing neutrophil responsiveness to proinflammatory agonists.

The sleep enhancer, melatonin, which is secreted during the night and during sleep, has multiple effects which include stimulation of GM-CSF and salivary IgA. Thus, it is hoped to observe a link with blood GM-CSF, melatonin, neutrophil activity and salivary IgA.

Also of interest for this study on sleep deprivation are the cytokines IL-2, linked with T cells; IL-3, which is a cell growth factor; IL-6, which is linked with cytotoxic T cells and with B cell differentiation); and IL-8, which is a chemoattractant for neutrophils.

Psychological Aspects of Fatigue

There is a considerable overlap of symptoms between chronic fatigue syndrome (CFS) and the overtraining syndrome (recently redefined as Unexplained Underperformance Syndrome (UUPS) Budgett et al., 2000). In addition to fatigue, these symptoms include lack of motivation, labile mood and a high incidence of minor infections. The latter suggests possible immunodepression in these individuals as a result of fatigue or mood changes. In a recent study on rowers receiving BCAA daily for one month during training, Hiscock and Castell (2001) found a small increase in lymphocyte proliferation. They also observed a significant effect in the alleviation of depression in those rowers taking BCAA compared with the placebo group, and a trend towards decreased fatigue. In elite cross-country runners, who were also U.S. Air Force Academy cadets, correlations have been observed between cognitive hardiness and immune cell numbers and function (Drummond et al., 2001). Some correlations with mood state changes were also observed. Thus the present study investigated whether there might be a link between fatigue and mood, resulting in immunodepression, which might occur as a result of just one night's sleep deprivation, and whether this could be ameliorated by the restoration of normal sleep or glutamine intervention.

METHODS

The fundamental approach used was:

- A fasting blood sample was drawn at 06:30-07:00 each morning for four consecutive mornings, Monday through Thursday.
- The blood was subjected to a complete blood count (CBC).
- After the first blood sample, the subjects experienced total sleep deprivation for approximately 36 hours, from Monday morning until Tuesday evening.
- One brief battery of cognitive performance tasks was employed on each of the four mornings, following the acquisition of the blood sample.
- One brief measure of simple cognitive performance was employed hourly throughout the night of total sleep deprivation.
- The subjects took daily oral doses of placebo ($n = 8$) or glutamine ($n = 8$), 2 x 5 g per day (double blind).
- Subjects were given standard, mixed meals while in the Sleep Lab, Days 1-3.

Male subjects (aged 21-40 yrs) were recruited from the local community and paid a standard rate per hour. Applicants were asked to avoid becoming subjects if they were aware of having clinical sleep problems, infectious conditions, immune compromises, and/or endocrine problems. The Brooks City-Base Institutional Review Board reviewed the experimental protocol and associated participant recruiting materials and methods. The U.S. Air Force Surgeon General approved the execution of the protocol (USAF/SG protocol number FB-2003-45-H). Each volunteer participant reviewed the experimental objectives and procedures, received answers to questions and signed an informed consent form.

Demographics

The following intake questionnaires were used. A *Demographics Questionnaire* was used to acquire information about age, height, weight, handedness (preferred), alcohol use, nicotine use, caffeine use, education level, and work history. In addition, the following instruments were used to acquire measures that could be used as covariates in statistical analyses.

Sleep Behaviour Questionnaire. The Sleep Behaviour Questionnaire dealt with adequacy and quality. It was designed by JC Miller and PA Hickey (2000), modified from Wylie et al. (1996), and (Hubinger, 1998). The subjects were asked, first, about participation in shiftwork. Second, about sleep length(s), sleep latencies and sleep inertia lengths on work days/nights. Third, to rate his/her "vulnerability to performance decrements at work" during eight 3-h periods of the day and night. Finally, subjects were asked about sleep length(s), sleep latencies and sleep inertia lengths on days/nights off.

Morningness-Eveningness. These two categories each account for approximately 15% to 20% of the human population and the mid-range category applies to the majority (60% to 70%) of humans. Morning types may be quite sensitive to delays in night sleep and their sleep duration during a morning sleep may be short. Morning types also may report low satisfaction with night work and may opt out of shift and night work. Evening types may tend more toward acceptance of night and shift work and thus may be the people who often go on to develop the kinds of health problems generally associated with night and shift work.

Sleep Hygiene and Practices Survey (SHAPS). The SHAPS acquired data concerning subjects' knowledge of the effects (1 to 7 scale) of selected daytime behaviour upon sleep and of the presence of caffeine in various over-the-counter medications, food and drink (Lacks & Robert, 1986).

Beck Depression Inventory. The Beck Depression Inventory is a 21-item self-report rating inventory measuring characteristic attitudes and symptoms of depression (Beck *et al.*, 1961). In general terms, a score of 0 to 3 is definitely normal and not depressed; 4 to 7 may be defined as normal or as mildly depressed; 8 to 15 is approximately moderately depressed; 16 to 24 is definitely clinically depressed; 25 to 30 is severely depressed; 31 and above requires immediate clinical evaluation.

Trait Anxiety Inventory. The State-Trait Anxiety Inventory (STAI) (Spielberger, 1983) differentiates between the temporary condition of "state anxiety" (see below) and the more general and long-standing quality of "trait anxiety" in young adults. Scores may range from 20 to 80. A higher score indicates higher anxiety. We acquired only the trait anxiety score.

Cognitive Hardiness Scale. Cognitive hardiness (CH) is a sense of control, commitment to the projects and people in one's life, and a tendency to appraise events as challenges versus threats (Kobasa et al., 1982). CH appears to moderate the relation between stress and both illness and depression, and has predicted cortisol reactivity. Scores range from 30-150. A higher score represents greater cognitive hardiness. The population mean is about 106, but in military groups it may be 114-118.

Epworth Sleepiness Scale. The Epworth Sleepiness Scale (ESS) (Johns, 1991, 1992) is a validated and reliable self-report measure of sleepiness, and the score was used as a potential covariate for analyses of performance data. The subjects use a number from 0 to 3 corresponding to the likelihood (never, slight, moderate, and high, respectively) that they would fall asleep in eight situations such as sitting and reading, watching TV. Ratings above 15 out of a possible 24 are cause for concern with respect to acceptable job performance.

Physiological Measures

Fasting, resting venous blood samples were taken at 6.30 a.m. for four days into lithium heparin (LiHep) vacutainers (Becton Dickinson), EDTA for cell counts, no anti-coagulant for hormones. The following parameters were measured: whole blood counts; plasma amino acid concentrations (enzymatic analysis of glutamine and free tryptophan); antioxidant capacity; cell cytokine production of IL-2, IL-3, IL-6, IL-8, GM-CSF.

In addition, the concentrations of plasma leptin, cortisol and caffeine levels were measured. Melatonin, IgA and cortisol levels were assessed in unstimulated saliva samples taken every two hours during the night's sleep deprivation in the Sleep Laboratory. Neutrophil function was monitored via oxidative burst.

Serum and urine samples were also measured for metabolic profiles using the new NMR technique of metabonomics (Nicholson et al., 2002). Metabonomics focuses on the analysis of metabolic processes and molecules, in contrast to genomics, which focuses on genes and gene products and proteomics, which deals with proteins. More specifically, metabonomics is the study of regulation patterns among low molecular weight metabolites to gain insight into the current metabolic status of an organism.

Assays

Glutamine: Plasma samples were deproteinized (Bernt & Bergmeyer, 1974), and plasma glutamine (p[Gln]) was determined enzymatically (Windmueller & Spaeth, 1974).

IL-2, IL-3, IL-6, IL-8, GM-CSF and Leptin: The cytokine concentrations were measured in samples from cell culture supernatant obtained with a modification of the technique of De Groote et al. (1992). Whole-blood samples from LiHep vacutainers were diluted 1/10 with glutamine-free RPMI-1640 medium, containing streptomycin/penicillin (1/1000). 1.8ml of diluted sample was pipetted into sterile 24-well flat bottom plates with/without the mitogen Concanavalin A (final concentration 50mg/ml), incubated at 37°C for 24 hrs (previously optimized in this group's Laboratory), centrifuged at 5-8°C, and the supernatant removed and stored at -20°C. The samples were measured using a quantitative sandwich enzyme immunoassay technique (ELISA, R&D Systems). Plasma concentrations of leptin were also measured using ELISA kits (R&D Systems).

Complete Blood Count. Circulating numbers of white blood cells (WBC) were measured (local Hospital, Becton Dickinson system), together with neutrophil, monocyte, lymphocyte numbers and differentials; platelet counts; haemoglobin (Hb), haematocrit (Hct), mean corpuscular volume (MCV); CD19⁺ B-lymphocyte cell counts; CD56⁺ Natural Killer cell counts. The Hospital Pathology Dept was unable to measure CD4/CD8 ratios as requested.

Caffeine: Plasma concentrations of caffeine were measured using a homogeneous enzyme immunoassay technique and the COBAS Mira Plus Clinical Analyser.

Antioxidant capacity: A novel chemiluminescent method was used to analyze antioxidant capacity via peroxynitrite and Vitamin E analogue (Knight Scientific Ltd, Plymouth, U.K.). This technique uses the photoprotein Pholasin[®], isolated from the bioluminescent mollusc *Pholas dactylus*. Pholasin[®] emits light when activated by free radicals and other reactive oxygen species such as those released by activated leukocytes.

Body Temperature. Oral temperature was taken repeatedly for 72 hours before the experiment and during the experiment. The subjects were instructed to refrain from eating or drinking for 15 min prior to scheduled temperature measurements, and proctors monitored this behaviour while the subjects were in the experiment.

Activity. The ActiWatch (Mini-Mitter Co. Inc., Oregon) wristwatch (WAM) is a small accelerometer which systematically recorded the individual's movement over time, both while awake and asleep, providing an effective means to identify sleep behaviour patterns outside the Sleep Lab. Subjects were instructed to wear the WAM on the wrist of the non-preferred hand and to wear it at all times except while showering or during water immersion. The actigraphy data were reduced using the Cole-Kripke sleep scoring algorithm (Cole *et al.*, 1992) to categorize each recorded epoch into sleep and awake periods. The WFC Activity Log was used to indicate for each half-hour of the three successive days before Day 1 whether subjects were sleeping or trying to sleep.

Incidence of Illness Questionnaire. An Incidence of Illness Questionnaire was given to the subjects on morning 4 of the study. Symptoms queried included cold, cough, sore throat, flu, diarrhoea, fever, headache, and a category for "other." Subjects were asked to mark whether or not they experienced any of the listed symptoms over the four-day study.

Performance tasks

Simple Cognitive Performance Battery. A cognitive performance test battery was implemented on desktop personal computers in the Windows® operating system using the Navy's Automated Neuropsychological Assessment Metrics (ANAM) library. This library of computerized tests was constructed to meet the need for measurement of cognitive processing efficiency in a variety of psychological assessment contexts that include neuropsychology, fitness for duty, neurotoxicology, pharmacology, and human factors research (Reeves *et al.*, 2001).

All stimuli were presented on the PC screen, and all performance task responses were made with the PC mouse buttons with the preferred hand. The battery included the following tests.

Simple response time task: rapid mouse-button press in response to the display of the * symbol. There were 20 trials, with an interstimulus interval that varied from 650 to 1100 msec. Timeout (no response) occurred at 1000 msec.

Mental arithmetic task: required a left or right click corresponding to a < 5 or > 5 solution of an addition-subtraction problem consisting of three single digits. The probe duration was set to 4500ms, with a timeout value of 5000ms. As soon as the subject responded another probe was presented. The task ran for three minutes.

Logical reasoning task: required a left or right click corresponding to a true-false choice about a positive or negative statement concerning the order of two symbols. The probe duration was set to 4500ms, with a timeout at 5000ms. As soon as the subject responded another probe was presented.

ANAM task training was given during the week immediately preceding Day 1. The subjects completed the ANAM battery six times during training, with two additional sessions on logical reasoning. The ANAM test order was Simple Response Time (SRT), Mental Arithmetic and Logical Reasoning.

Measures acquired from these tasks included percent accuracy, mean response time for correct responses (MNRTC), numbers of omissions, standard deviation of response time for correct responses (SDRTC), and throughput. Throughput is a measure that includes aspects of both speed and accuracy in a single score. It was computed as the number of correct responses per minute:

$$\#Correct \times (60,000 / \text{mean } RT_{ALL})$$

Vigilance Performance. Vigilance performance was assessed using the Psychomotor Vigilance Task (PVT), an extension of the Unprepared Simple Reaction Time Task (Dinges, 1992; Dinges *et al.*, 1997; Ambulatory Monitoring, Inc., Ardsley NY). This task is learned quickly (two 1-min trials) and is sensitive to fatigue due to sleep loss, circadian variation, and shift work. The portable, battery-operated device ran a continuous, simple response time test for 10 min. The task required sustained attention and discrete motor responses: the subject watched a digital counter (LED) and, when the counter started to run, turned it off as quickly as possible with a button press using the preferred hand. The interstimulus interval varied from 2 to 10 sec. A relatively quick response was about 200 msec. Timeout (no response; a lapse) occurred at 500

msec. Subjects completed 10-min PVT once during training in the week immediately preceding Day 1.

The variables provided by the PVT-192 included the number of lapses, the mean of the reciprocals of all response times (MnRRT), the mean of the reciprocals of the 10% fastest response times (MnFRRT), the mean of the reciprocals of the 10% slowest response times (MnSRRT), and the slope of the reciprocal response times during the 10-minute test. The number of lapses was transformed by PVT software from the expected Poisson distribution to an approximately normal distribution by a square root transform: $[\sqrt{\text{lapses}} + \sqrt{\text{lapses} + 1}]$.

Vertical Jump. Participants performed repeated standing vertical jumps (Wiklander & Lysholm, 1987), using a Probotics timing mat and cable transducer to measure jump height and ground contact time. Vertical jump performance is a standardized measurement for explosive leg power and has been shown to be sensitive to sustained operations.

Subjective measures

Stanford Sleepiness Scale. To use the Stanford Sleepiness Scale (SSS; Hoddes *et al.*, 1973), the subject selects one of seven sets of Likert-scale descriptors, ranging from 1, “Feeling active and vital; alert; wide awake,” to 7, “Almost in reverie; sleep onset soon; lost struggle to remain awake.” The SSS usually correlates with standard measures of performance and usually reflects the effects of sleep loss. The Profile of Mood States vigour scale has also demonstrated sensitivity and reliability with respect to quantifying perceptions of sleepiness.

State Anxiety Inventory. The State Anxiety Inventory (STAI; Spielberger, 1983) is designed to evaluate feelings of apprehension, tension, nervousness, and worry, which increase in response to physical danger and psychological stress. The self-report inventory consists of 20 items. Scores may range from 20 to 80. A higher score indicates higher anxiety. The state anxiety norm for working men, aged 19 to 39 yr, is 36.54 +/- 10.22 (*ibid.*).

Mood changes. The Profile of Mood States (POMS) was used to measure dimensions of affect or mood. It consists of 65 adjectives describing feeling and mood to which the subjects responded according to scale ranging from 0-5 for severity, and is summed up under six mood

factors: tension, depression, anger, vigour, fatigue, confusion. Vigour-Activity and Fatigue-Inertia factors were used for this study.

PROCEDURES

The subjects started each experiment by fasting overnight at home (20:30-06:30) and then giving a blood sample seated (#1, 20 ml) at the Sleep Lab at 06:30. They completed one set of ANAM cognitive tasks, the SSS and the POMS, and ate breakfast in the lab. In a pilot study, Experiments 1 and 2, they then spent Day 1 at work or home, and returned to the Sleep Lab by 18:00, without their vehicle.

After an 06:30 fasting blood sample (#2), they completed one set of ANAM cognitive tasks, the SSS and the POMS, and ate breakfast in the lab. For all experiments on Day 1 the subjects ate dinner in the Sleep Lab and then remained awake all night with fasting (20:30-06:30). Oral temperature and psychomotor vigilance task (PVT) data was acquired hourly all night.

In Experiments 1 and 2, they then spent Day 2 recovering from sleep loss at home or at work. In Experiment 3, they stayed in the Sleep Lab for all of Day 2 and slept in the Sleep Lab that night. The following two mornings, after an 06:30 fasting blood sample (#3, #4) at the CASL, they completed one set of ANAM cognitive tasks, the SSS and the POMS, ate breakfast in the lab and were released. The subjects' recovery sleep patterns were monitored by actigraphy across the 48 h starting at 06:30 on morning 2.

Supplemental glutamine

Nutritional glutamine was purchased from Oxford Nutrition, Witney, UK. They also provided an identically packaged placebo (maltodextrin). Subjects were given 2 x 5g doses of glutamine/placebo dissolved in water, one in the morning and one in the evening, in the Sleep Lab. Random allocation was made via computer programme operated by someone who had no connection with the study.

Statistics

Generally, the daily, early-morning data were subjected to a 2-factor, 2- x 4-level analysis of variance (ANOVA) with repeated measures on factor B. Factor A was Group (Placebo, Glutamine) and Factor B was Day (1 through 4). Significance levels for repeated measures were

adjusted by the Greenhouse-Geisser (GG) method. Analyses were conducted with the BMDP statistical software package (version 7, program 2V; SPSS). Probability values were adjusted for the effects of repeated measures using the Greenhouse-Geisser method. *Post hoc* assessments were made with the Neuman-Keuls Studentized Range Statistic (Winer, 1971).

The multiple, repeated measures taken while the subjects were in the laboratory were subjected to between-group, multiple, 2-tail t tests without correction. The expected sample size of 8 provided a test power of 0.46 for a 2-tail test for an effect size of 1 standard deviation unit with a confidence level of 0.95 and independent groups (Cohen, 1988).

Statistical significance was accepted at the 90% level of confidence ($p < 0.10$) in this exploratory study. Variabilities are reported here as +/- one standard deviation.

RESULTS

Demographic measures

Sleep Behaviour Questionnaire: All fifteen subjects were day workers; one worked a fixed day shift. All of the following parametric values were assessed for inter-group differences by multiple, 2-tail t tests. The subjects reported sleeping a total of 5.50 to 8.00 h on work nights (mean 6.57 \pm 0.75 h), with sleep latency for the major, nocturnal sleep period ranging from 5-45 min (mean 15.9 \pm 11.1 min). Four subjects reported napping on workdays. The 15 subjects reported their ideal average sleep length for work nights as 7.09 \pm 0.84 h. Thus, they may have incurred a sleep deficit of about (7.09 – 6.57 h =) 31 min per night.

The median values for the subjects' reports of vulnerability to performance decrements at work during eight 3-h periods of the day are shown in Figure 1. They displayed the classic pattern of high vulnerability in the midnight-to-dawn hours with a mid-afternoon modulation in the circadian cycle.

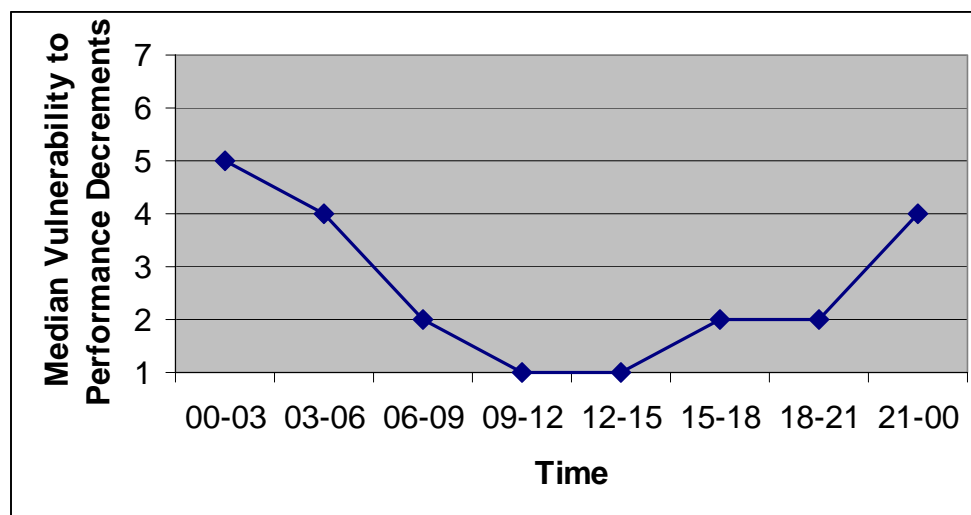


Figure 1. Median values for reports of vulnerability to performance decrements at work during eight 3-h periods of the day.

The two Groups differed in their reported sleep lengths on days/nights off. The Placebo Group reported sleeping 7.14 \pm 0.69 h, while the Glutamine Group reported sleeping 8.71 \pm 2.00 h. ($p = 0.07$, close to significance). The combined Groups reported their sleep latency for the major, nocturnal sleep period as ranging from 5 to 30 min (mean 13.21 \pm 6.96 min). Six subjects reported napping on days off. The two Groups also differed significantly ($p(t) = 0.05$) in

their reported ideal sleep lengths on days/nights off. The Placebo Group reported an ideal length of 7.57 +/- 0.79 h, while the Glutamine Group reported sleeping 8.93 +/- 1.46 h. Thus, they may have incurred sleep deficits of about (7.57 – 7.14 h =) 26 min and about (8.93 – 8.71 h =) 13 min per day/night off, respectively.

Morningness-Eveningness: Morningness-eveningness scores were distributed relatively evenly across Groups, as shown in Table 1. The mean scores of the two groups did not differ significantly ($p(t) = 0.45$).

Table 1. Morningness-eveningness scores.

	Definitely Morning	Moderately Morning	Neither
Placebo	1	2	5
Glutamine	1	1	5

Sleep Hygiene and Practices Survey (SHAPS): Scores on the sleep hygiene (SH) knowledge section ranged from 15 to 33, a higher score (up to 39) indicating less sleep hygiene knowledge. Scores on the caffeine knowledge (CK) section ranged from 55.55 to 100; a higher score (up to 100) indicating better knowledge of caffeine. Scores on the sleep practices section ranged from 7 to 41, a higher score (up to 133) indicating less healthy sleep hygiene practice. The mean scores for the two Groups (Table 2) indicated average sleep hygiene knowledge, good caffeine knowledge and good sleep hygiene practices. The two Groups differed somewhat on caffeine knowledge, with the Placebo Group demonstrating slightly better knowledge ($p(t) = 0.09$).

Table 2. Mean scores (+/- 1 sd) on the SHAPS (* $p < 0.10$).

	SH score	CK score*	SP score
Placebo	20.8 +/- 3.1	89.4 +/- 11.9	19.5 +/- 5.3
Glutamine	24.0 +/- 6.6	77.4 +/- 13.8	25.1 +/- 10.6

Beck Depression Inventory: Five of the eight placebo subjects and five of the seven glutamine subjects produced a score of 0 to 3: definitely normal and not depressed (Table 3). One in each group produced a score of 4 to 7 (normal or mildly depressed); two placebo subjects scored 8 to 15 (moderately depressed) and one glutamine subject scored 16 to 24 (clinically depressed).

Table 3. Beck Depression Inventory scores.

	0-3	4-7	8-15	16-24
Placebo	5	1	2	0
Glutamine	5	1	0	1

Trait Anxiety Inventory: The average trait anxiety score ranged from 33 to 49. The two Groups did not differ significantly ($p(t) = 0.27$). The grand mean anxiety score was 44.3 ± 4.2 . The trait anxiety norm for working men, aged 19 to 39 yr, was 35.55 ± 9.76 . Thus, these subjects displayed higher than average trait anxiety, just under one standard deviation above the mean. Those who exceeded 1 sd above the normal mean were relatively evenly divided between the two groups, with 4 of the 8 placebo subjects and 3 of the 7 glutamine subjects falling slightly more than 1 sd above the mean.

Cognitive Hardiness Scale: The Cognitive Hardiness scores ranged from 91 to 141. The two Groups did not differ significantly ($p(t) = 0.80$). The grand mean score was 117.8 ± 12.3 , in agreement with the reported range for means in military groups: 114 to 118.

Epworth Sleepiness Scale: The ESS scores for the two groups did not differ significantly ($p(t) = 0.72$). The grand mean score for all subjects was $7.0 \pm \text{sd } 2.8$ (range 2 to 11). Thus, there appeared to be no occurrences of self-reported excessive daytime sleepiness among the subjects.

Pre-Experimental Circadian Rhythm in Oral Temperature: Repeated measurements of oral temperature taken during the 72 hours pre-experiment were subjected to cosinor analysis using the methods described by Naitoh et al. (1985) and Koukkari et al. (1974) and by Faure et al. (1990). The parameter values of the rectilinear component of 72-h trends did not differ significantly between the two Groups (by 2-tail t tests) except for the proportion of total variance accounted for by the rectilinear component of change. The grand mean oral temperature for the 72 h was 97.50 deg F with a $+0.06 \text{ deg F per day}$ grand mean slope. The proportion of variance accounted for by the rectilinear estimate was significantly smaller for the Pla Group (6.4%) than for the Gln Group (16.7%; $p(t) = 0.08$). This difference was due primarily to two Glutamine subjects whose temperatures were changing at the rates of $+0.6 \text{ deg F per day}$ [284] and $-0.6 \text{ deg F per day}$ [285], respectively.

Two subjects in the Pla Group and one subject in the Gln Group had very flat, uninterpretable cosinor curve fits. For the rest of the subjects, the parameter values of the cosinor component of 72-h trends did not differ significantly between the two Groups (by 2-tail t tests) except for the time of acrophase. The grand mean cosine (half-wave) amplitude for the 72 h was 0.49 deg F with the cosine curve accounting for a grand mean of 19.9% of the variance in the residual temperatures (after removal of the rectilinear trend).

PHYSIOLOGICAL MEASURES

Complete Blood Count

White blood cell counts (10/uL). There was a significant effect of Day on neutrophil count ($F(3,39) = 2.73$, $MSe = 0.177$, $p(GG) = 0.079$). There were no other significant effects of Group or Day on the cell counts for neutrophils, lymphocytes or monocytes. The grand means of the counts were 3.46 (53%), 2.28 (35%) and 0.54 (8%), respectively. The normal ranges are about 45 to 74%, 16 to 45% and 4 to 10% of white blood cells, respectively, so these means fell within the normal ranges.

Visual inspection of the white blood cell counts for the individual components revealed a similarity for the pattern of change across days; specifically, a slight leucocytosis immediately following the night of sleep deprivation (Fig. 2). This pattern was true for the significant Day effect on neutrophils and also reflected somewhat reliably in terms of the total white blood cell count: There was a significant effect of Day on the count ($F(3,39) = 3.04$, $MSe = 0.294$, $p(GG) = 0.0517$; Figure 2). A *post hoc* assessment with the Neuman-Keuls procedure indicated that the total white blood cell count was 7.5% higher on Days 2 and 3 than on Day 1, though only ($p < 0.10$). There was no significant effect of Group on WBC count. In the pilot study an interesting trend for basophil numbers was observed in the glutamine group. Unfortunately repeat data was not available in the present study since the cell counts were erroneously measured by the operator to zero decimal places only.

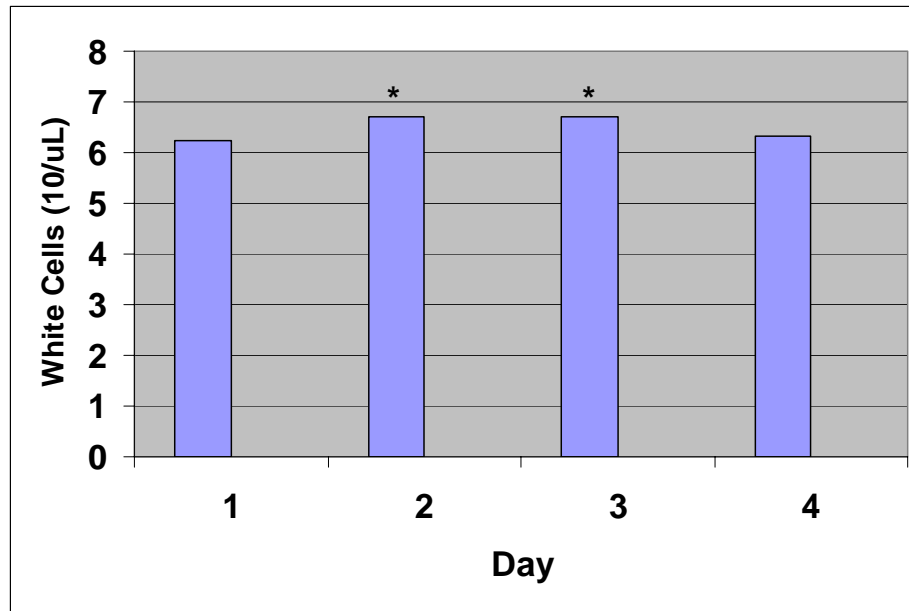


Figure 2; *The effect of Day on total white blood cell count: higher (7.5%) on Days 2 and 3 than on Day 1 (* $p < 0.10$, close to significance)*

Haemoglobin (Hb; g/dL) and haematocrit (Hct; %). There was no significant effect of Group on Hb or Hct, nor significant Group-Day interactions. There was a significant effect of Day on the Hb concentration ($F(3,39) = 15.46$, $MSe = 0.146$, $p(GG) < 0.001$; Figure 3). A *post hoc* assessment with the Neuman-Keuls procedure indicated that the Hb concentration was significantly higher (about 5%) on Day 3 than on Days 1, 2 and 4 ($p < 0.10$). Haematocrit showed a similar pattern, increasing gradually across the four Days ($F(3,39) = 15.62$, $MSe = 1.311$, $p < 0.001$). Thus, there appeared to be a slight tendency for haemoconcentration across the four Days.

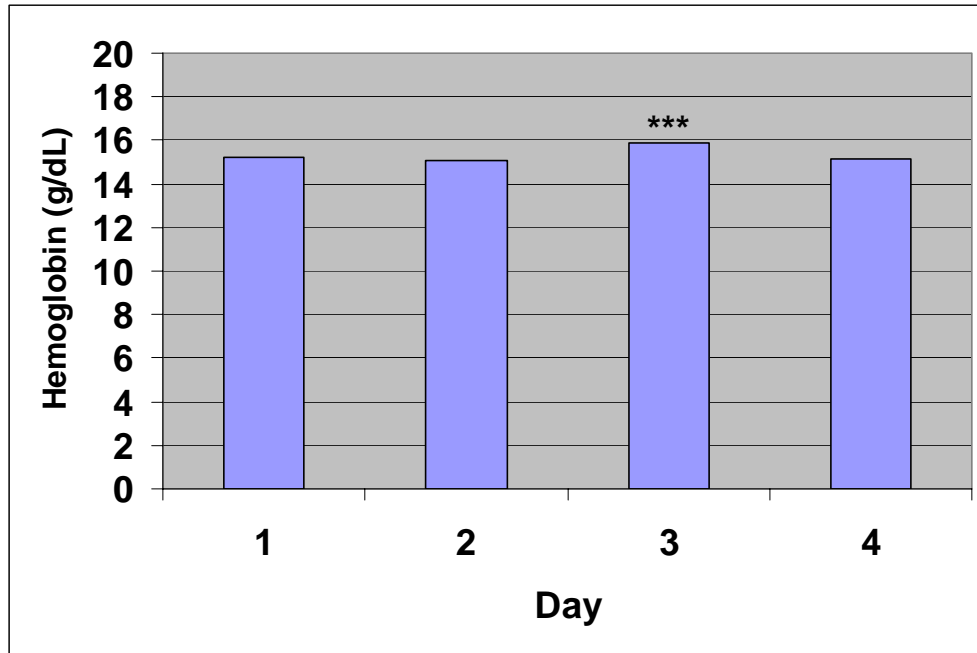


Figure 3: *The effect of Day on haemoglobin concentration: significantly higher (5%) on Day 3 than on Days 1, 2 and 4 (***)* ($p < 0.01$)

Mean Corpuscular Volume (MCV; in fL): There was no significant effect of Group on MCV, nor Group-Day interaction. There was a significant effect of Day on MCV ($F(3,39) = 7.17$, $MSe = 0.307$, $p(GG) = 0.002$; Figure 4). A *post hoc* Neuman-Keuls assessment indicated that MCV was significantly higher (about 1%) on Days 2, 3 and 4 than on Day 1 ($p < 0.01$ and 0.05 , respectively). Thus, while there was a statistically reliable effect of Day on the volume of individual red blood cells, the size of the difference was probably not very meaningful and may have been related to the mild haemoconcentration noted above.

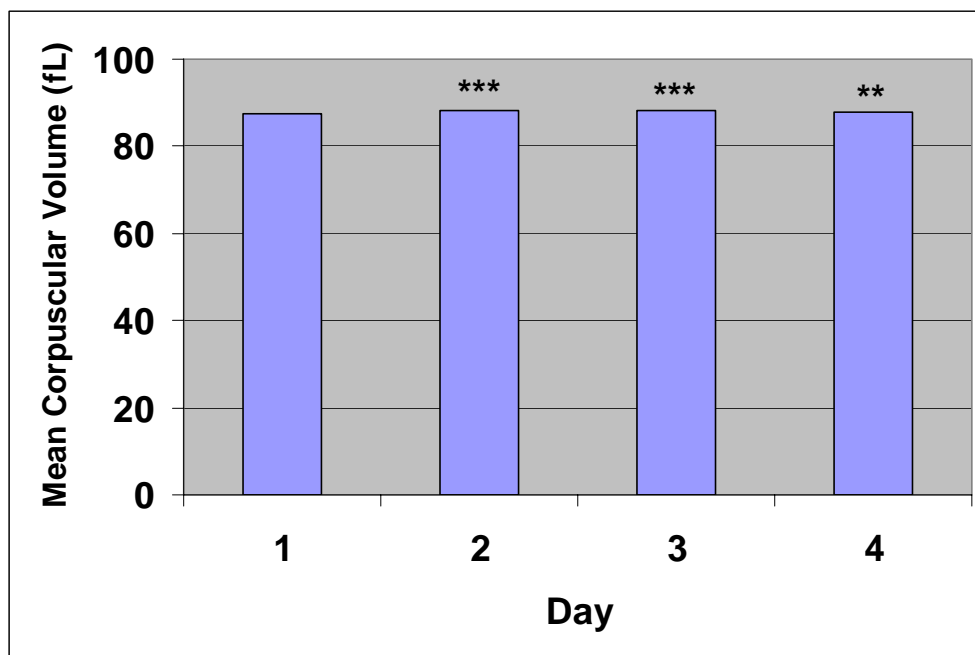


Figure 4: *The effect of Day on mean corpuscular volume: significantly higher (1%) on Days 2, 3 and 4 than on Day 1 (*** $p < 0.01$, ** $p < 0.05$)*

Platelet count (10⁹/L). The effect of Group was not significant. There was a significant effect of Day ($F(3,39) = 2.66$, $MSe = 69.77$, $p(GG) = 0.073$) and a significant Group-Day interaction effect ($F(3,39) = 3.31$, $MSe = 69.77$, $p(GG) = 0.0389$) on the platelet count (Fig. 5). A *post hoc* assessment with the Neuman-Keuls procedure indicated that the platelet count for Days 2, 3 and 4 in the Placebo Group was significantly higher (about 8%) than on Day 1 for the Placebo Group and for all 4 days of the Glutamine Group.

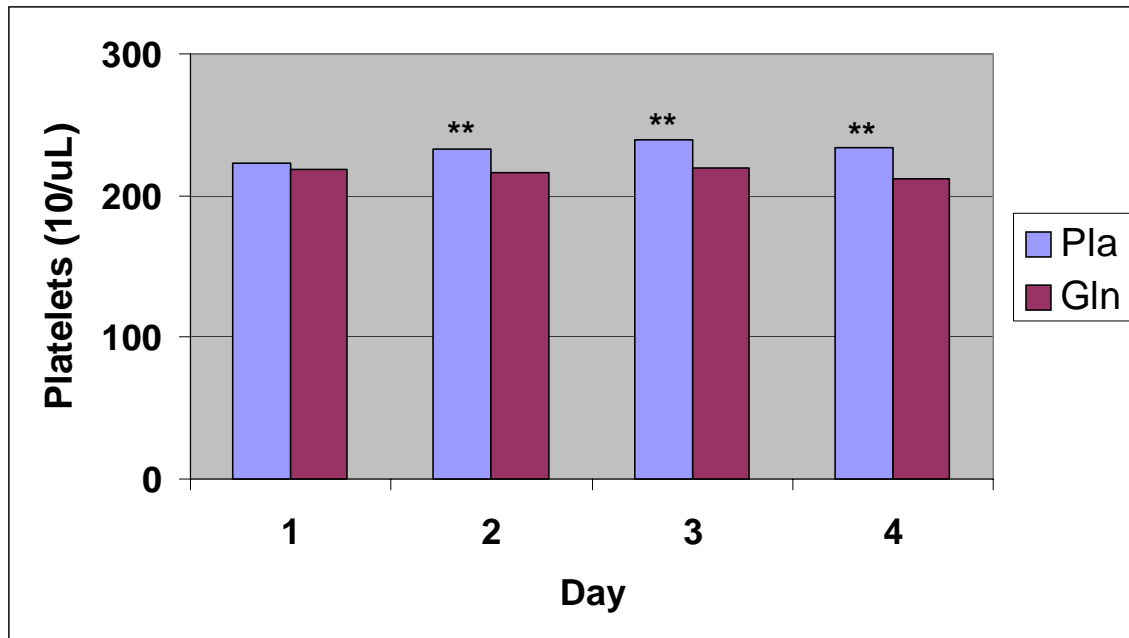


Figure 5: *The interactive effects of Group and Day on platelet count: significantly higher (8%) for Pla Group Days 2, 3 and 4 cf. Day 1 and Gln Group Days 1 to 4 (** $p < 0.05$)*

CD19⁺CD3⁻ B-lymphocyte cell counts. There were no significant effects of Group or Day on this measure.

CD56⁺CD3⁻ Natural Killer cell counts (NK). The effect of Group was not significant. There was a significant effect of Day ($F(3,39) = 2.93$, $MSe = 4674.2$, $p(GG) = 0.073$) and a significant, interactive effect of Group and Day ($F(3,39) = 2.57$, $MSe = 4674.2$, $p(GG) = 0.098$) on this measure (Fig. 6). A *post hoc* assessment with the Neuman-Keuls procedure indicated that the combined count was significantly higher (about 46%) for Pla Group Day 3 than all other Pla Days and for Gln Days 2 and 4 ($p < 0.10$). Thus, it appeared that the night of sleep deprivation followed by a night of recovery sleep might have caused a sharp elevation in this combined cell count in the Pla Group but not in the Gln Group.

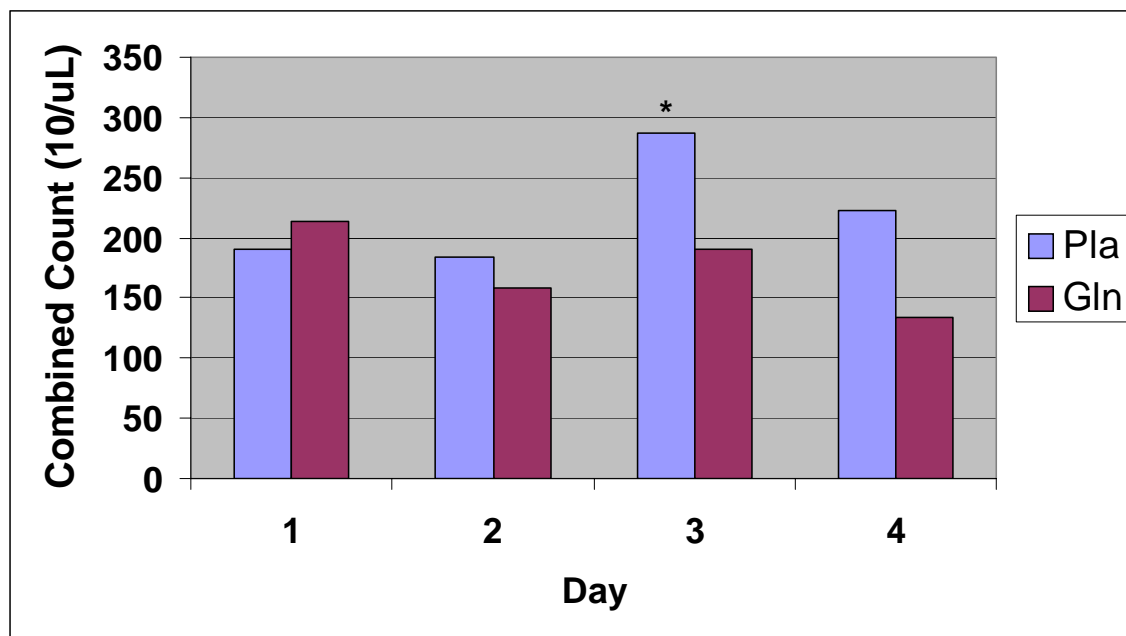


Figure 6: The interactive effects of Group and Day on $CD56^+CD3^-$ Natural Killer cells: significantly higher (46%) for Pla Group on Day 3 than all other Pla Group Days and Gln Group Days 2 and 4 (* $p < 0.10$)

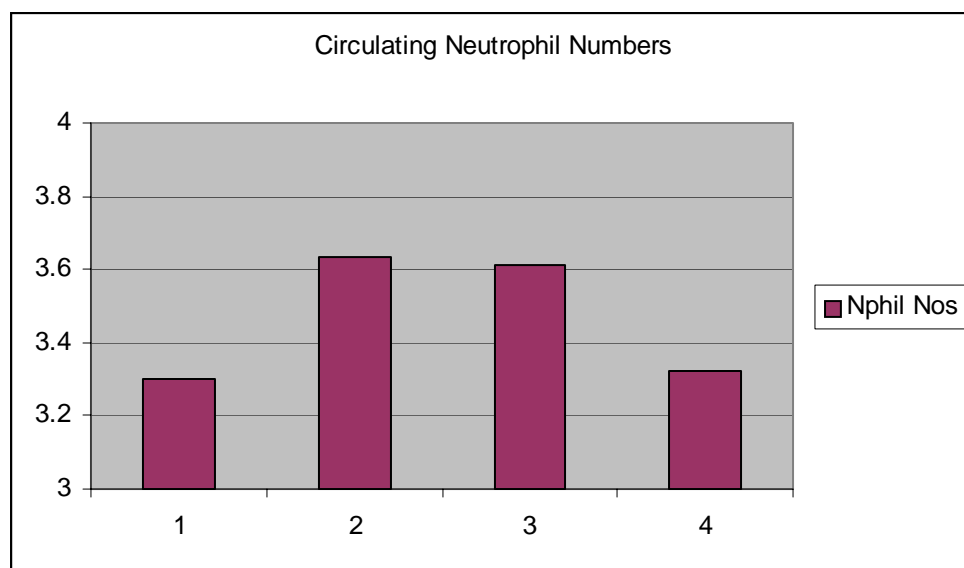


Figure 7: Circulating neutrophil numbers ($\times 10^9/L$). Close to significance increase ($p < 0.067$) at Day 2 compared with baseline. No difference between Groups.

Neutrophil oxidative burst: These cells were PMA stimulated, fixed with paraformaldehyde and transported to the Veterans Administration Hospital, San Diego where there is considerable expertise in FACScan analysis which was necessary to count the fluorescently labelled cells as mean fluorescence intensity per cell. At first sight it appeared that oxidative burst was increased on Day 2. However, it became clear during subsequent data analysis that there were viability problems with some batches of samples during the preparation (cause unknown) and this led to inconsistencies in the data (data not shown) which rendered the results unsuitable for further analysis.

Glutamine

There were no significant differences in p[Gln] between the Pla and Gln groups on any of the days ($p>0.05$). There was a non-significant trend ($p<0.119$) for a difference in p[Gln] between the groups on day 4.

There were no significant changes in p[Gln] in either group over the four days, $p>0.05$. However, whereas on Day 2 p[Gln] was maintained in the Gln group, there was a non-significant 5% decrease in the Pla group. There was a trend ($p<0.092$) for p[Gln] to be lower on Day 4 compared with Day 3 in the Pla group. On Day 4, compared to baseline, p[Gln] in the Pla group was 13.9% lower (see Fig. 8).

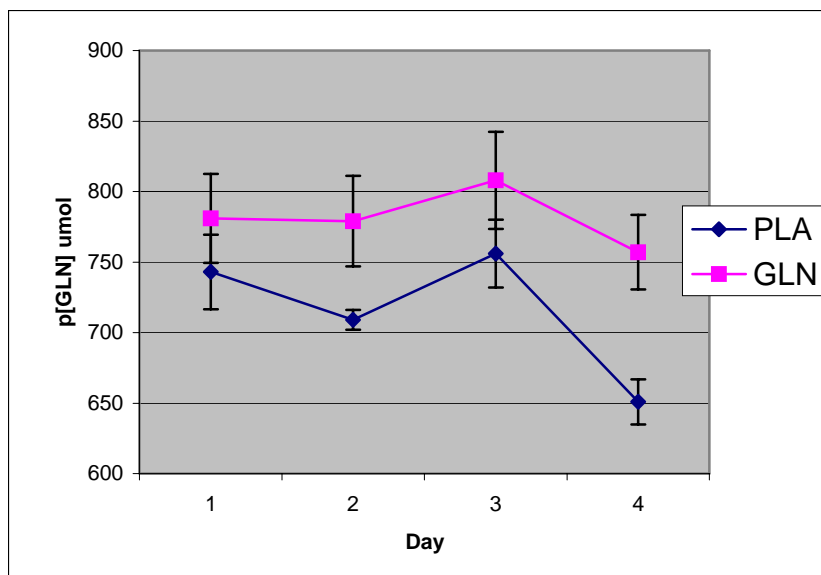


Figure 8: The effect of Day on p[Gln] M, means \pm SEM

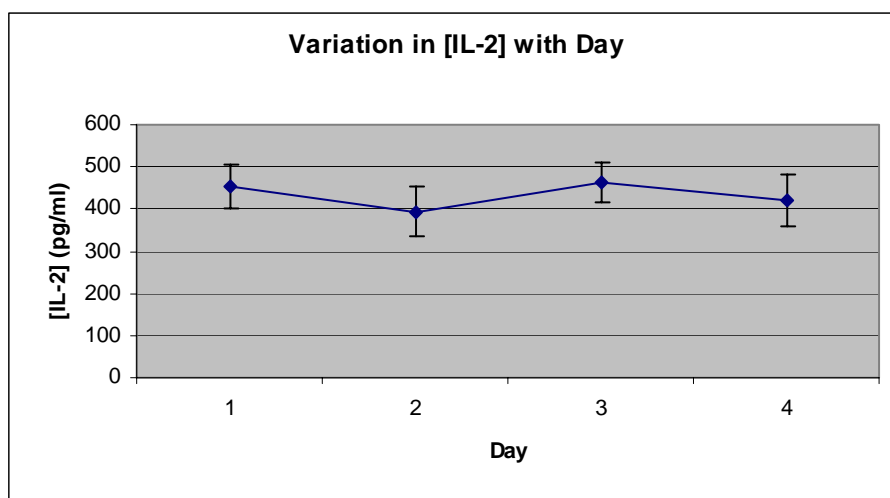


Figure 9: Effects of Day on IL-2 production for all subjects, means \pm SEM

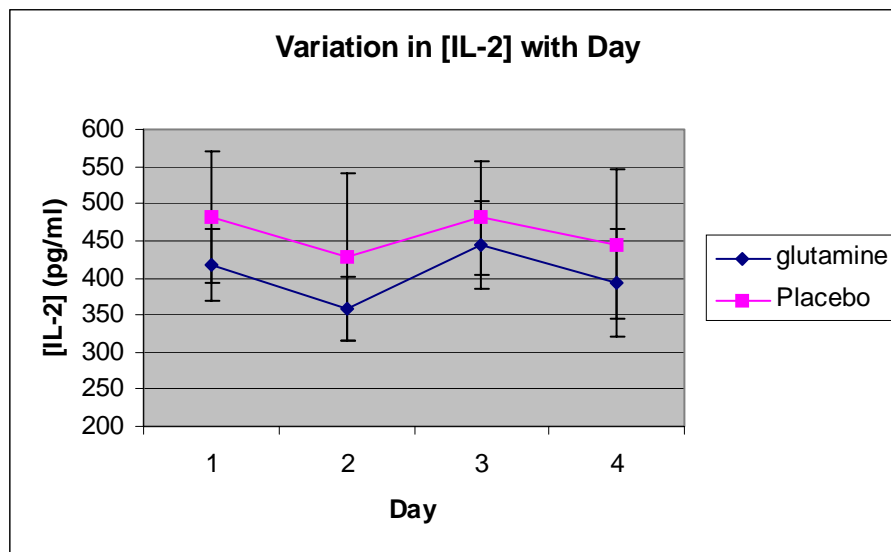


Figure 10: The effect of Day on IL-2 production. Gln Group vs. Pla Group, means \pm SEM

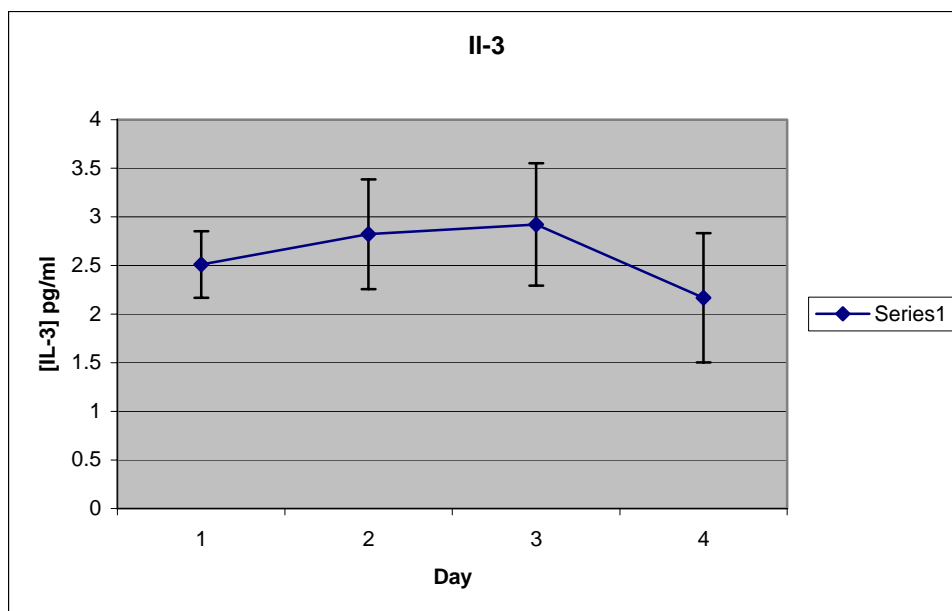


Figure 11: The effect of Day on IL-3 production for all subjects (NS), means \pm SEM

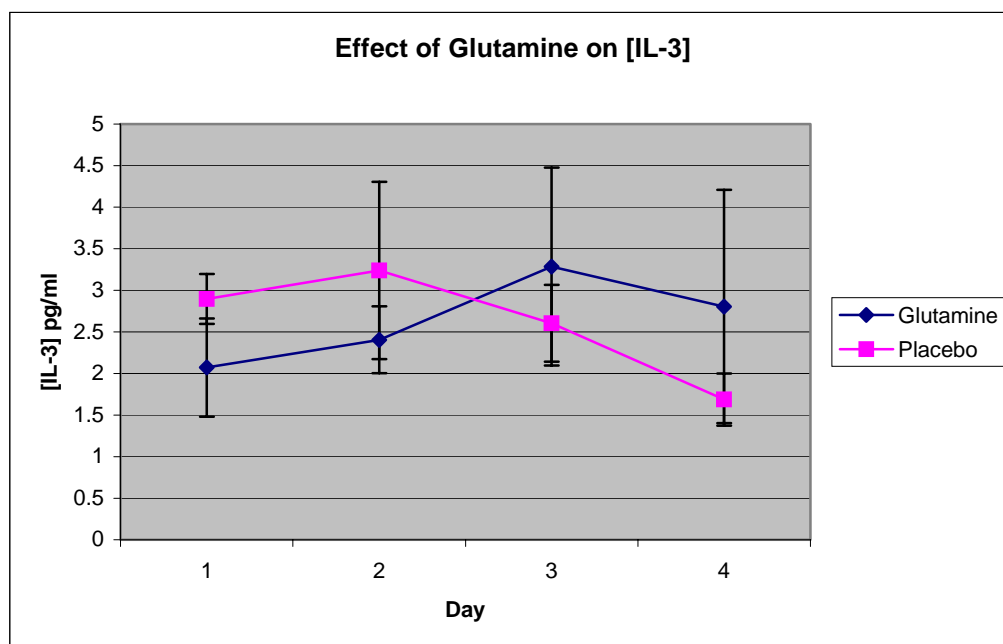


Figure 12: The effect of Day on IL-3 production. Gln Group vs. Pla Group (NS), means \pm SEM

IL-3

There were no significant effects of Day or Group in IL-3 production, largely due to the high inter-individual variation, though there were some non-significant trends towards an increase at Day 3 (Fig. 11) and a decrease at Day 4. IL-3 production appeared to increase in the Pla Group at Day 3 (NS), and decrease in the Gln Group at Days 3 and 4 (NS).

There were no significant differences in IL-6 production between days in the Pla group ($p > 0.05$). IL-6 production remained at baseline on Day 2 and there was a non-significant 17.3% increase on Day 3. There was a close to significant ($p < 0.069$) 18.3% decrease in IL-6 production from Days 3 to 4, but IL-6 production on Day 4 was not significantly different to baseline. There was a significant effect of Day on IL-6 production in the Gln group ($p < 0.05$). IL-6 production was decreased 35.6% ($p < 0.019$) on Day 2 compared to baseline; there was a close to significant ($p < 0.051$) increase on Day 3 to return levels to baseline where they remained.

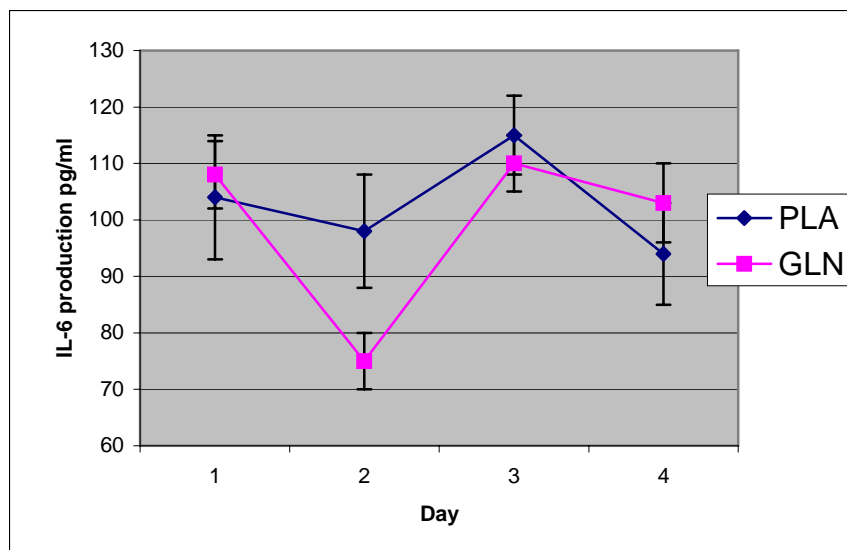


Figure 13: The effect of Day on IL-6 production pg/ml, means \pm SEM

One subject (290) displayed a pattern for p[IL-6] over the four days that was the extreme opposite of the pattern for most other subjects. For this reason the analyses were repeated without including this subject's data.

IL-6 Analyses without Subject 290

There were no significant differences between groups on any of the days ($p > 0.05$). The pattern of change for both groups over the four days was similar (see Fig. 14).

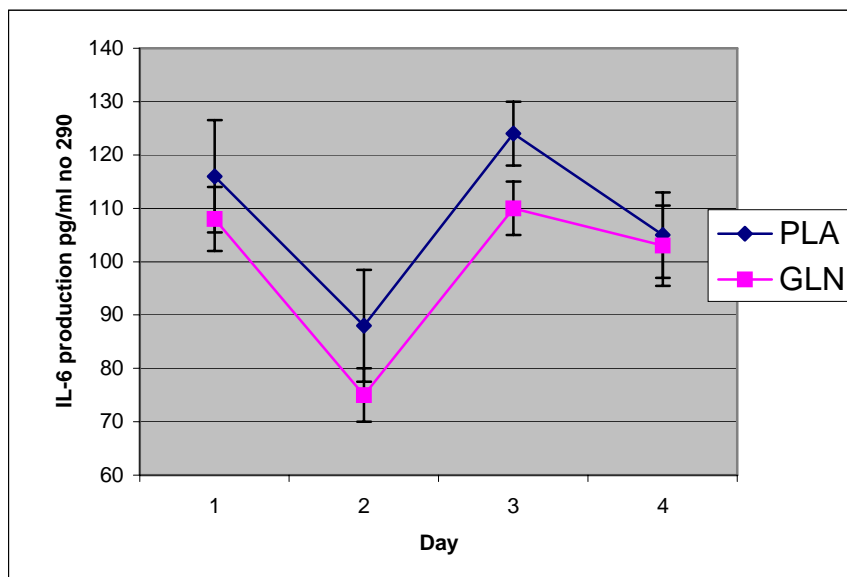


Figure 14: The effect of Day on IL-6 production Without Subject 290, means \pm SEM

There was no effect of Day on IL-6 production in the PLA group when subject 290 was removed ($p>0.05$). However, on Day 2 IL-6 production was decreased 24.1% compared to baseline, then increased 40.9% on Day 3 to reach slightly above baseline levels, then decreased 15.3% on Day 4 to end at a level slightly below baseline (all non-significant changes). The GLN group was unaffected by elimination of this subject.

IL-8

There was no significant effect of Day on IL-8 production in either group ($p>0.05$). There was a non-significant trend for a 7.3% decrease in the Pla Group and 10% increase in the Gln Group in IL-8 production from Days 1 to 2. In the Pla Group, IL-8 production did not deviate significantly from this level for the remainder of the study. In the Gln Group, IL-8 production returned to baseline on Day 3 and remained at this level.

Similar to IL-6 production, the same subject in the Pla Group displayed a pattern for IL-8 production that was the extreme opposite to the norm for that group. The analyses were conducted again with this subject removed.

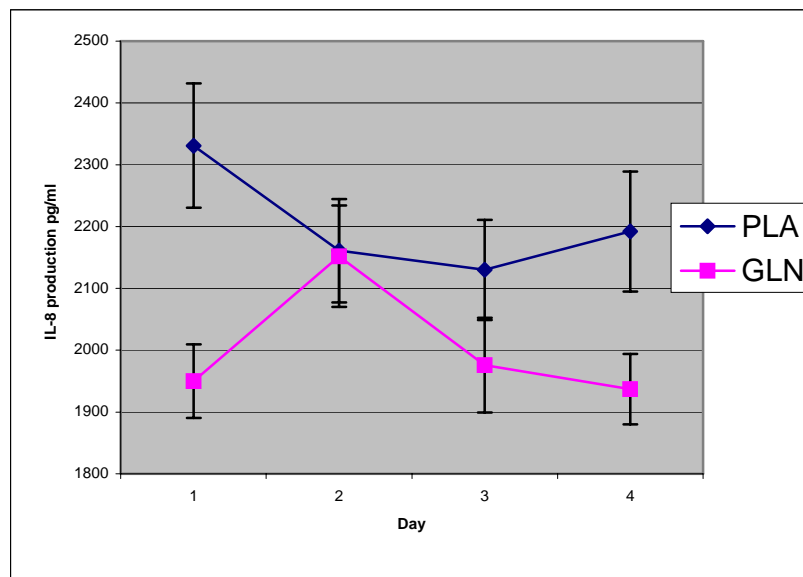


Figure 15: The effect of Day on IL-8 production pg/ml, means \pm SEM

IL-8 Analyses without Subject 290

There were no significant differences between the groups on any of the days ($p>0.05$). However, there were trends for difference between the groups on Days 1 and 4, which were close to significance ($p<0.058$ and $p<0.106$ respectively; see Fig. 16).

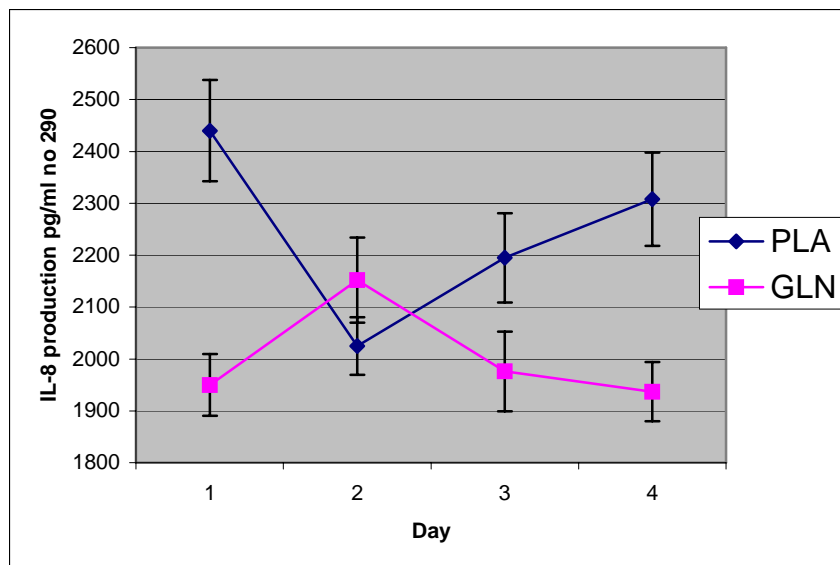


Figure 16: The effect of Day on IL-8 production pg/ml without Subject 290, means \pm SEM

There was no significant effect of Day on IL-8 production in the Pla Group with subject 290 removed ($p>0.05$). However, there was a near significant ($p<0.096$) trend for IL-8 production to be decreased 17% on Day 2 compared to baseline. It was increased 8.4% from Days 2 to 3, then a further 5.1% from Days 3 to 4 to finish 5.4% below baseline levels (all non-significant).

GM-CSF production

For all subjects combined there was a difference in GM-CSF levels between Days 2 and 3 ($p<0.05$), and close to significance between Days 1 and 2 ($p<0.1$), and 3 and 4 ($p<0.15$). There were no significant differences between GM-CSF levels in the glutamine and placebo groups.

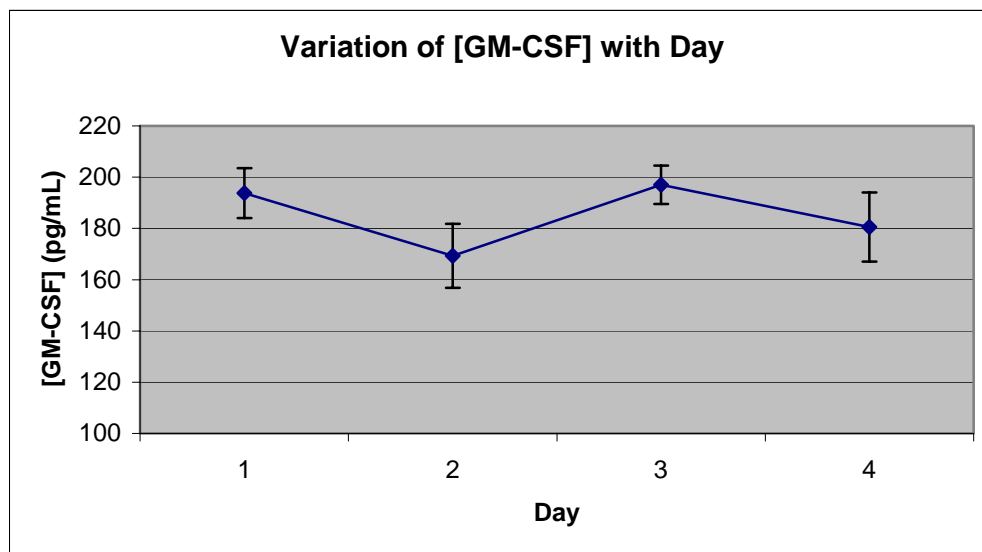


Figure 17: The effects of Day on GM-CSF production in all subjects, means \pm SEM

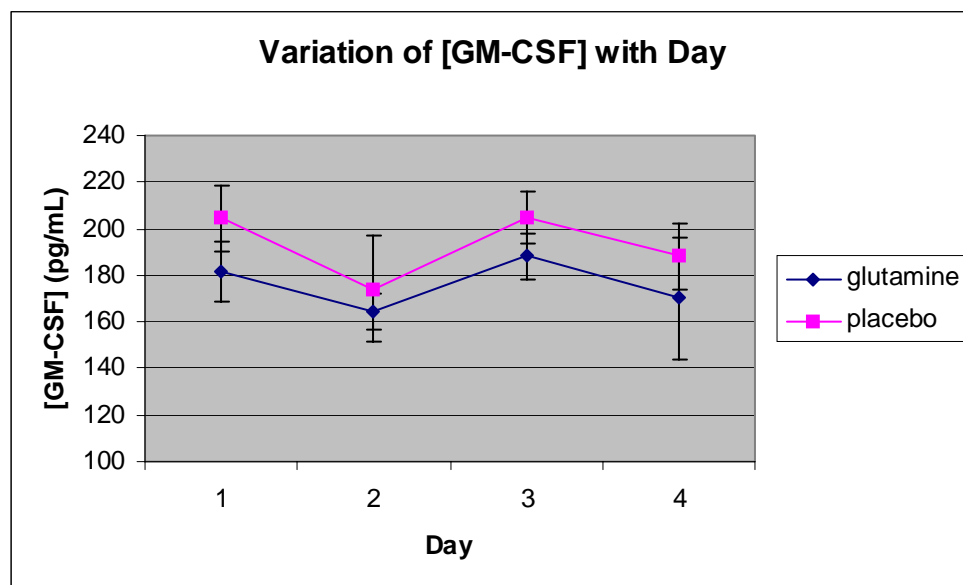


Figure 18: The effects of Glutamine vs. Placebo on GM-CSF production, means \pm SEM

Leptin

There were no significant differences between the groups on any of the days ($p > 0.05$; see Fig. 19). There was no significant effect of Day on p[Lep] in either group ($p > 0.05$). There was a great deal of subject variation in p[Lep] over the four-day study with a baseline range of 0.21-4.61 ng/ml for both groups. In the Pla Group, baseline range was 0.54-4.61 ng/ml; four subjects

had higher p[Lep] on Day 2 (of these three were over 20%), and four had lower p[Lep] on Day 2 (all were 10% or more lower) compared to baseline. On Day 4, four subjects had increased p[Lep] (of which three were over 40%), and three subjects had decreased p[Lep] (all 10% or more) compared to baseline.

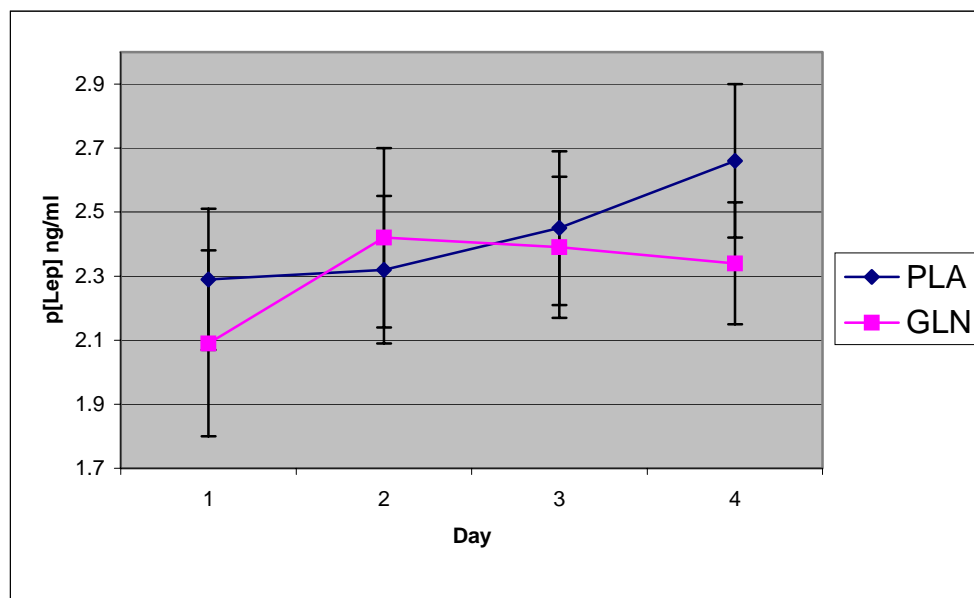


Figure 19: The effect of Day on p[Lep] ng/ml, means \pm SEM.

In the Gln Group, baseline range was 0.21-3.98 ng/ml. On Day 2, four subjects had increased p[Lep] (of these three were over 20%), two had decreased p[Lep] (one was over 40%), and one subject maintained p[Lep] compared to baseline. On Day 4, four subjects had higher p[Lep] (all over 15%), and three subjects had lower p[Lep] (one over 30%) compared to baseline. As a result of such variation, p[Lep] would be better considered on an individual basis.

Caffeine

There were no significant differences in p[Caff] between groups on any of the days, $p > 0.05$.

However there was a non-significant trend ($p < 0.12$) for a difference between the groups on Day 4 (see Fig. 20).

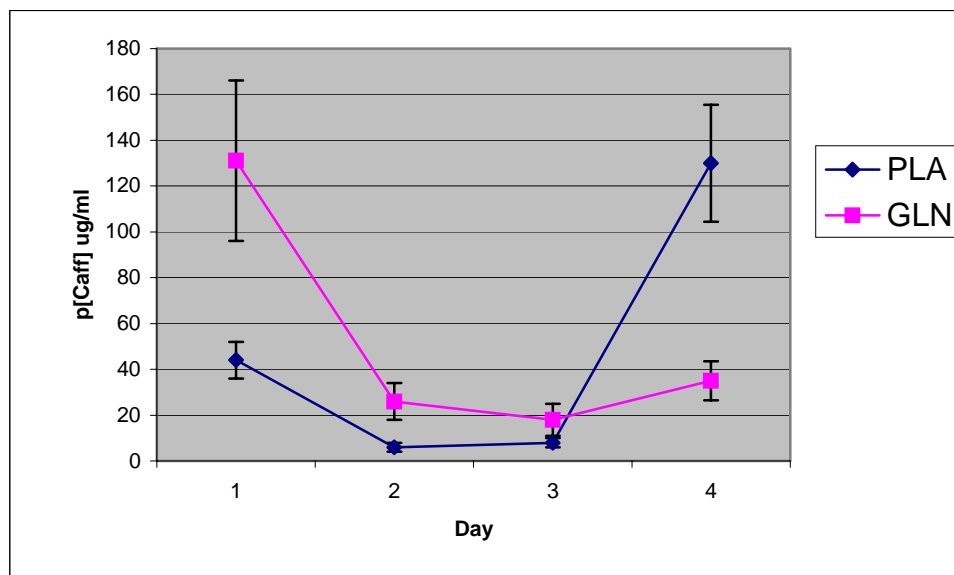


Figure 20: The effect of Day on p[Caff] g/ml, means \pm SEM

There was a significant effect of Day on p[Caff] in the Pla group ($p < 0.05$), and no significant effect of Day on p[Caff] in the Gln group ($p > 0.05$). In the Pla group, p[Caff] was significantly ($p < 0.027$) decreased by 88.6% on Day 2 compared to baseline, and significantly ($p < 0.042$) increased 16-fold from Days 3 to 4. The apparent decrease in p[Caff] at Day 2 in the Gln group of 80.2% was not significant. On Day 3, all subjects apart from one in the Gln group had p[Caff] below 30 g/ml. In the Pla group, p[Caff] was over 100 g/ml in 4/7 subjects on Day 4, compared to only 1/7 in the Gln group. The latter subject maintained p[Caff] at over 80 g/ml for the duration of the study. None of these subjects reported a caffeine score per day of > 2 , which corresponds with the average number of caffeinated drinks (50mg per drink) consumed daily.

Cortisol

There were no significant differences in salivary (s[Cort]) between groups on any of the days, $p > 0.05$. However, there was a non-significant trend towards an increase on Day 4 (Fig. 21). Plasma cortisol, on the other hand, showed a significant difference between the Groups, with the glutamine Group having 24% higher cortisol on Day 4 than the placebo group ($p < 0.001$).

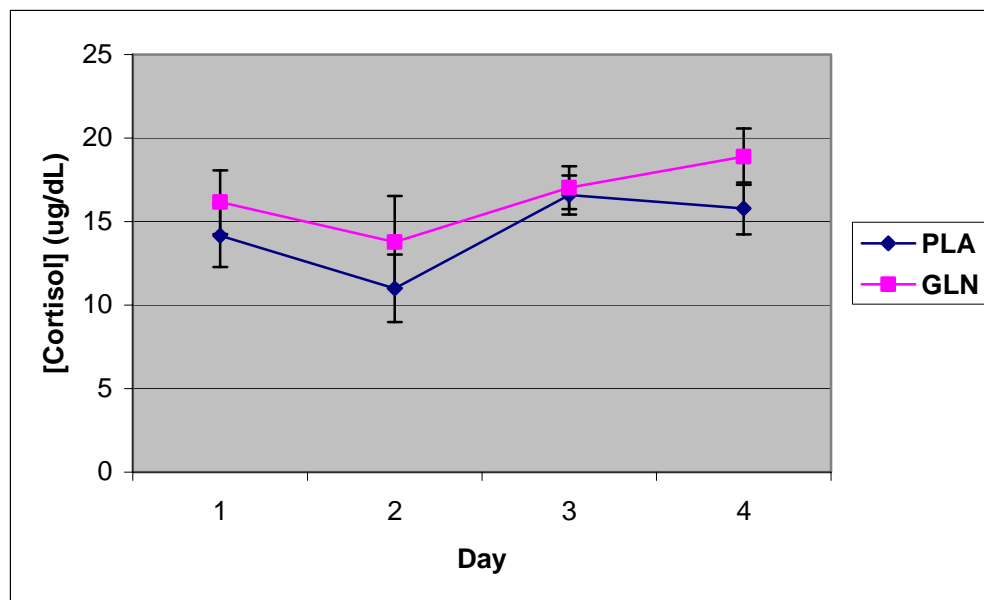


Figure 21: The effect of Day on salivary cortisol (s[Cort]) g/dl, means \pm SEM

Metabonomics

In an initial examination of the data it has been possible to see differences in the urine samples but not in plasma samples. In the urine samples, there was clear increase in urea, hydroxypyruvate and methylhistidine on Days 2, 3 and 4 compared with Day 1. For the same time points there was a decrease in urinary creatinine. With regard to additional analysis of the metabonomics data, further, more complex analysis is required to assign identities to a few other metabolites in which some relatively small changes have also been observed.

Salivary IgA

There was a progressive increase in salivary IgA for all subjects combined, which began on Day 2 after one night's sleep deprivation. There was a significant difference between the groups on Day 2, with Pla Group being 17% higher than the Gln Group ($p < 0.05$), and a trend towards the Gln Group being 29% higher than the Pla Group at Day 4 (close to significance, $p < 0.10$).

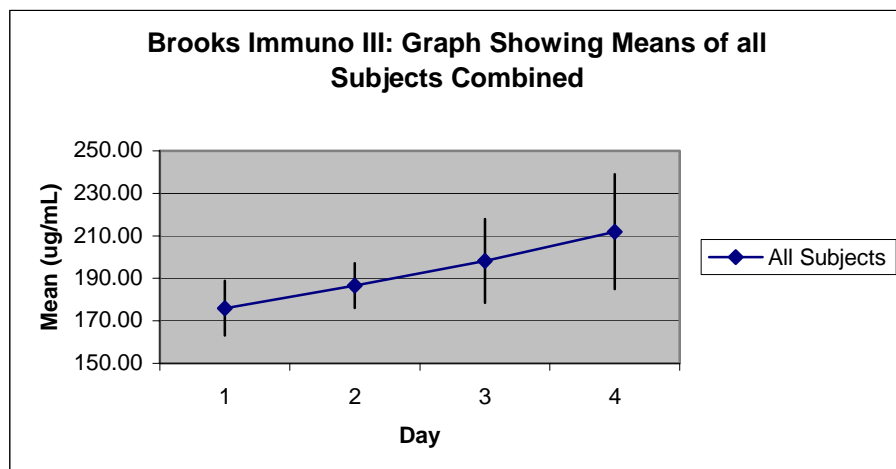


Figure 21A: Effect of Day on salivary IgA for all subjects combined. Means +SEM

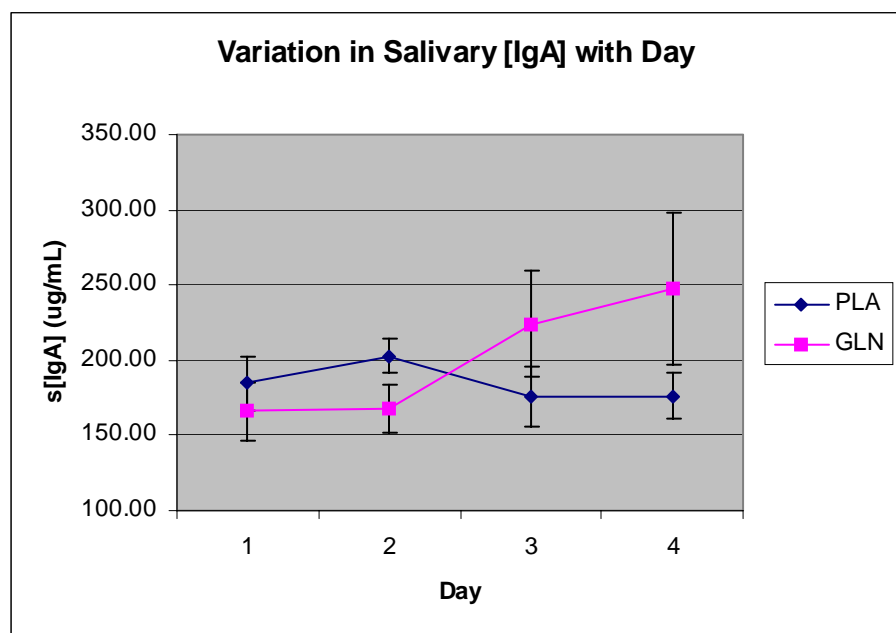


Figure 21B: Effect of Day on salivary IgA for Gln Group vs. Pla Group. Means \pm SEM

Melatonin

Analysis of the samples for melatonin has not yet been completed due to laboratory relocation. Data should be available in March 2005.

Plasma antioxidant capacity

Plasma antioxidant capacity (as measured by Vitamin E analogue equivalent) demonstrated in general a good level of fitness in these subjects. There was a non-significant trend towards a decrease on Day 2. The data has not yet been assessed for a difference between Pla and Gln.

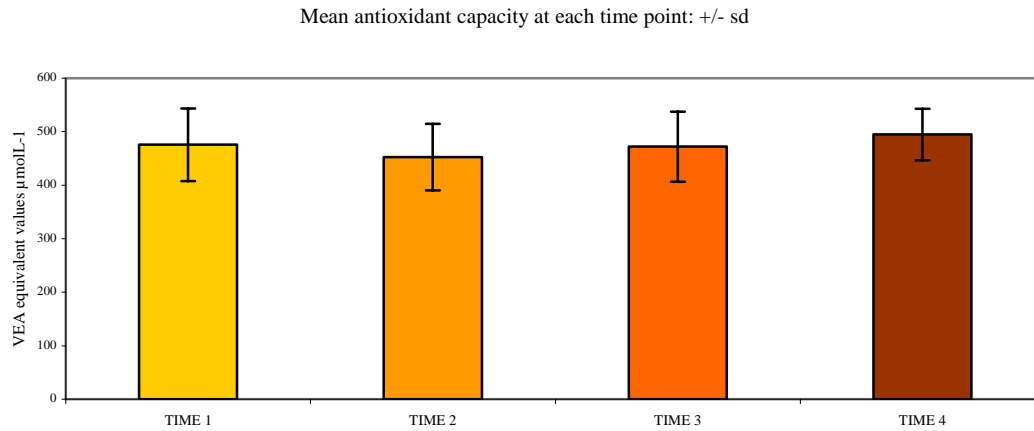


Figure 21C: The effects of Day (Time) on plasma antioxidant capacity. Means \pm SD. Data for Pla vs. Gln Groups have not been assessed.

Oral temperature

Oral temperature displayed the classic circadian pattern (Fig. 22): temperature peaked in the late-afternoon-to-early-evening hours and reached a nadir during the pre-dawn hours during the night of sleep deprivation. Multiple, 2-tail t tests revealed no statistically significant differences between Groups across all of the measurement periods. A 2- x 3-level, 2-factor ANOVA for the effects of Group and Day revealed no significant effect of either factor across the first three mornings of the study.

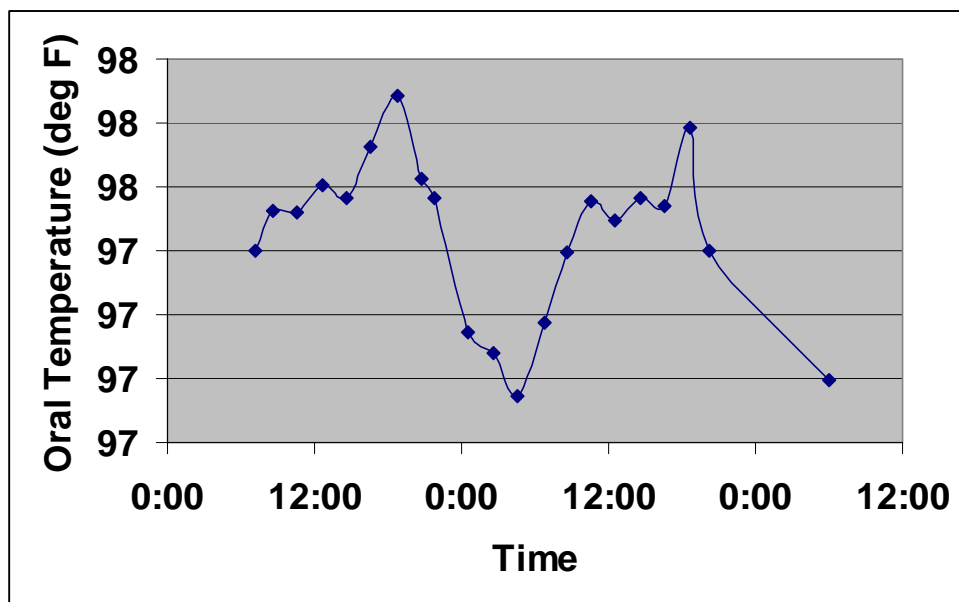


Figure 22: 3-day plot of mean oral temperature (n = 15).

In terms of linear trends, there were no statistically significant differences (by 2-tail t tests) between the two Groups. The grand mean oral temperature during the study was 97.45 ± 0.41 deg F with a grand mean slope of -0.13 deg F per day. Neither of the subjects whose temperatures were changing at $+0.6$ and -0.6 deg F per day before the experiment showed extraordinary temperatures or slopes during the study.

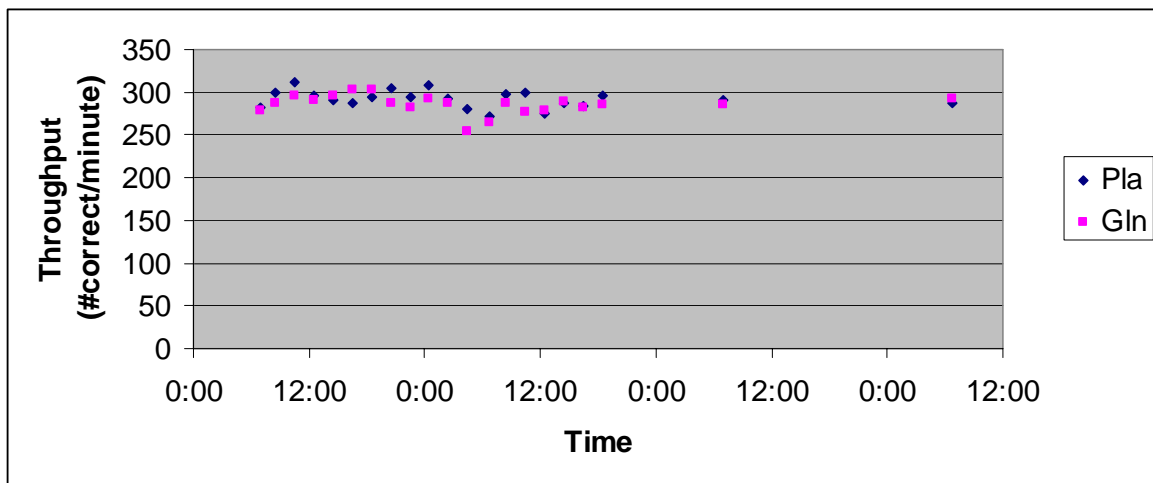
For the 72 hours following the experiment, the two Groups did not differ significantly (by 2-tail t tests) in mean oral temperature nor in day-to-day change. The grand mean, post-experimental temperature was 97.56 ± 0.45 deg F and the grand mean slope was 0.08 deg F per day, nearly identical to the pattern observed for the 72 hours before the experiment.

Incidence of Illness Questionnaire and Post-Experimental Oral Temperature: There was a pre-experimental incidence of illness in one subject in the Placebo Group who reported sneezing due to allergies. One Placebo subject reported a cough and sore throat on the mornings following each night of recovery sleep (Days 3 and 4). Another Placebo subject reported a runny nose on the morning of Day 3. Neither of these subjects was the one who reported allergy problems before the study. One Glutamine subject reported upper respiratory congestion on the morning of Day 1, and one reported sinus pressure on the morning of Day 3. Thus, it appeared that two Placebo subjects and one Glutamine subject may have experienced some upper

respiratory symptoms as a result of the night of sleep deprivation. There was poor compliance with post-experimental reporting of incidence of illness, but no positive reports occurred.

PERFORMANCE MEASURES

Simple Response Time: The throughput of the Placebo and Glutamine Groups did not differ significantly at any test time ($p > 0.10$; Fig. 23). The use of the throughput value here (instead of simple response time) corrected for the occasional occurrence of a missed signal.



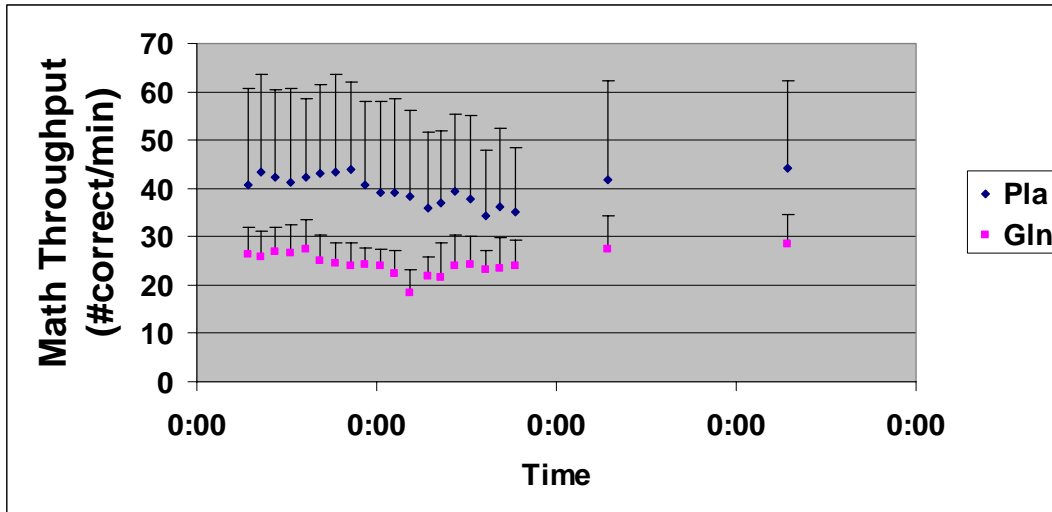


Figure 24: 4-day plot of Mental Arithmetic throughput scores (± 1 SD). Pla Group > Gln Group all Days except 06:30 Day 3; multiple t tests ($p < 0.05$ < 0.10)

Since the two Groups differed significantly, in general, and since we had 21 observations on each subject, we also assessed the data as within-subject standard scores. In this data set, this approach to standardization reduced relative intersubject variability by 29%, increased relative intrasubject variability by a factor of 2.6, and reduced the relative intergroup difference by 69%. (Details for effects of the standardization on this data set are shown in Appendix I). The standardized data produced an easily-recognizable picture of performance decrement during the pre-dawn hours of the night of sleep deprivation (Fig. 25). There were no statistically significant differences between Groups in the standardized data set.

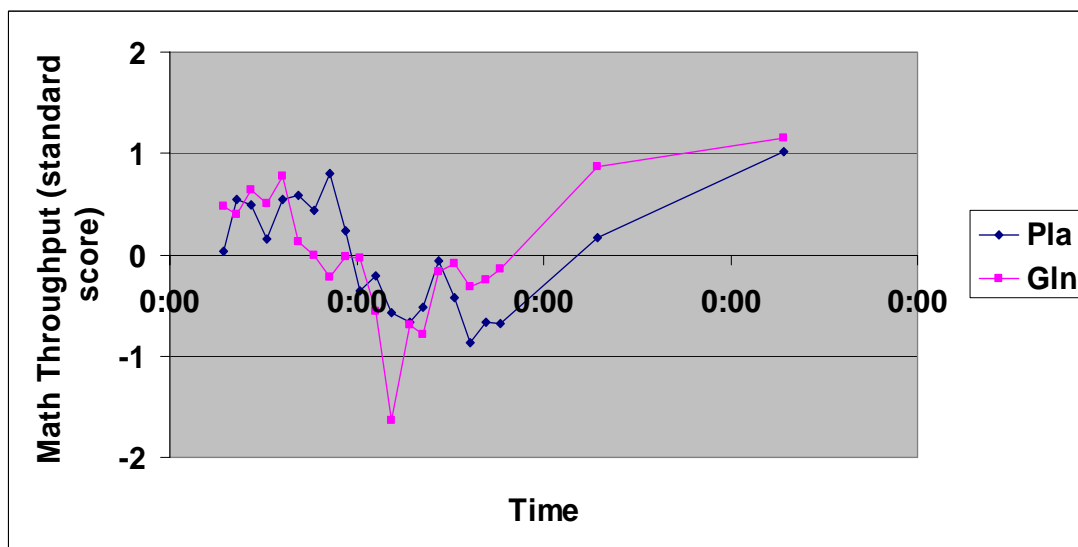


Figure 25: 4-day plot of Mental Arithmetic throughput standard scores.

A 2- x 4-level ANOVA was then applied to Mental Arithmetic throughput standard scores. The Gln Group performed better than the Pla Group ($F(1,13) = 5.00$, $MSe = 0.296$, $p=0.044$). Re-calculated in terms of grand mean and grand SD of the data set, both groups performed at ca.39.6 and 34.8 corr/min, respectively, a 14% performance advantage for the Gln Group (Fig. 26).

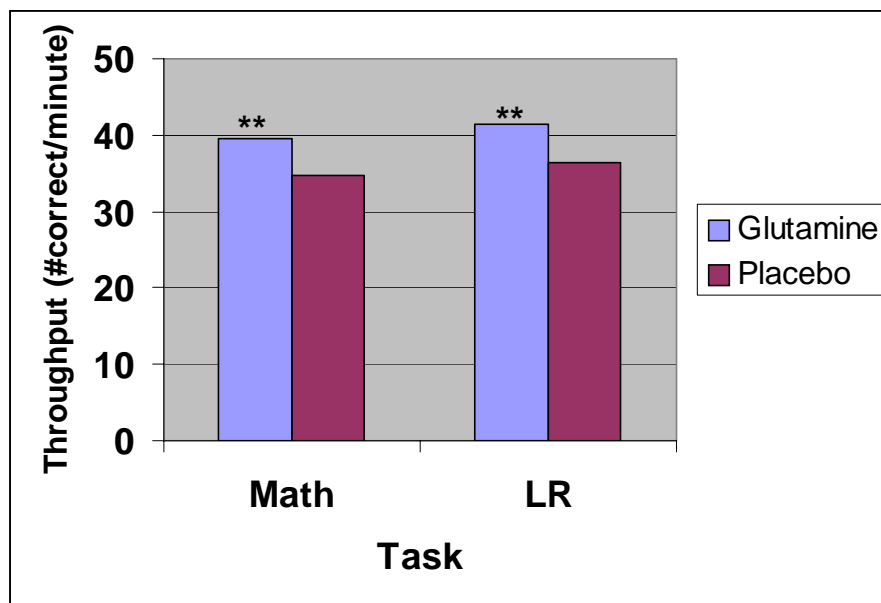


Figure 26: Group effect on re-calculated Mental Arithmetic and Logical Reasoning throughput: higher throughput for the Gln Group (** $p<0.05$).

There was a significant effect of Day on standard scores ($F(3,39) = 8.78$, $MSe = 0.920$, $p=0.0008$; Fig. 27) but no significant Group-Day interaction. *Post hoc* assessment of Day effect indicated that throughput was significantly higher on Days 1, 3 and 4 than on Day 2 ($p<0.05$; $p<0.01$).

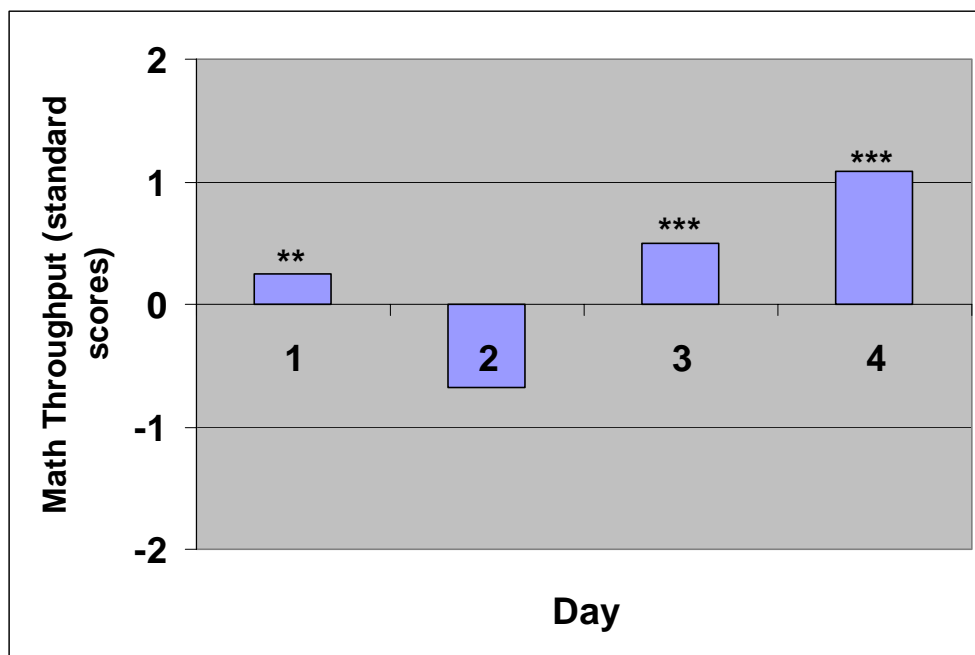


Figure 27: The effect of Day on Mental Arithmetic throughput standard score: throughput was significantly higher on Days 1, 3 and 4 than on Day 2 (** $p < 0.05$, *** $p < 0.01$).

Logical reasoning

The throughput of the Placebo Group was slightly greater than that of the Glutamine Group at 04:30 and 08:30 on morning 2 ($p(t) < 0.10$; Fig. 28).

To provide parallelism with the analysis of the Mental Arithmetic task data, within-subject standard scores for Logical Reasoning were also examined and an overnight pattern similar to the Arithmetic difference scores was found (Fig. 29). Statistically significant differences occurred at 04:30 on the night's sleep deprivation (Pla Group > Gln Group) and on the morning of Day 3 (Gln Group > Pla Group; $p < 0.05$).

A 2- x 4-level, 2-factor ANOVA was then applied to Logical Reasoning throughput standard scores. As with the Mental Arithmetic task, the Gln Group performed better than the Pla Group ($F(1,13) = 5.38$, $MSe = 0.857$, $p = 0.037$). Re-calculated in terms of grand mean and grand standard deviation of the data set, the two groups performed at about 41.4 and 36.4 corr/min, respectively, again a 14% performance advantage for the Gln Group (Fig. 28, above).

There was also a significant effect of Day on the difference score ($F(3,39) = 5.55$, $MSe = 0.857$, $p(GG) = 0.0073$; Fig. 30) but no significant Group-Day interaction. *Post hoc* assessment

of the Day effect indicated that throughput was significantly higher on Days 1, 3 and 4 than on Day 2 ($p<0.05$ and $p<0.01$). This appears to have been a fatigue effect on both Groups.

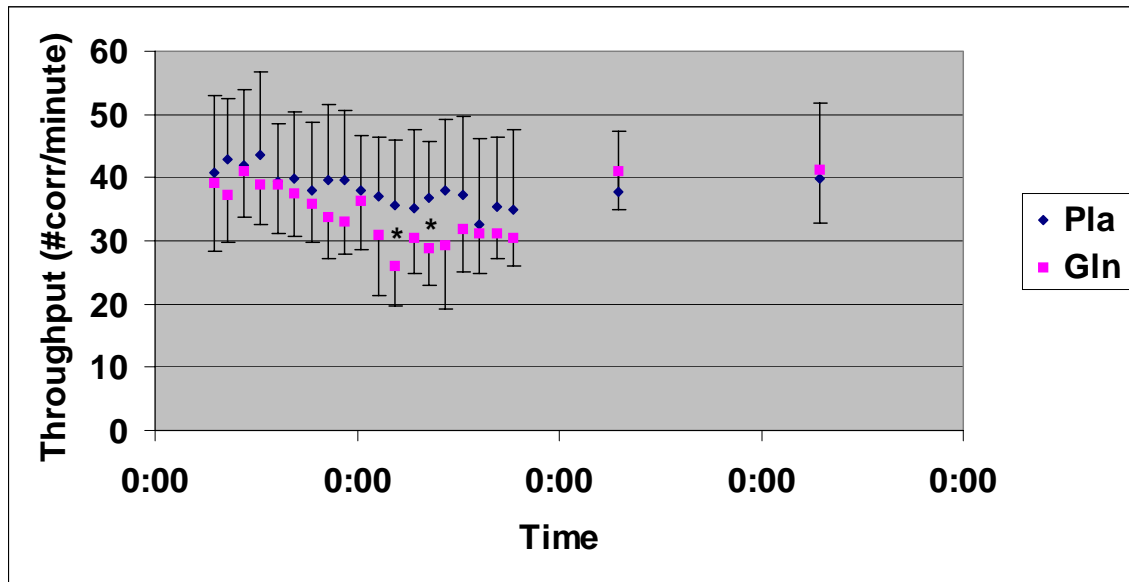
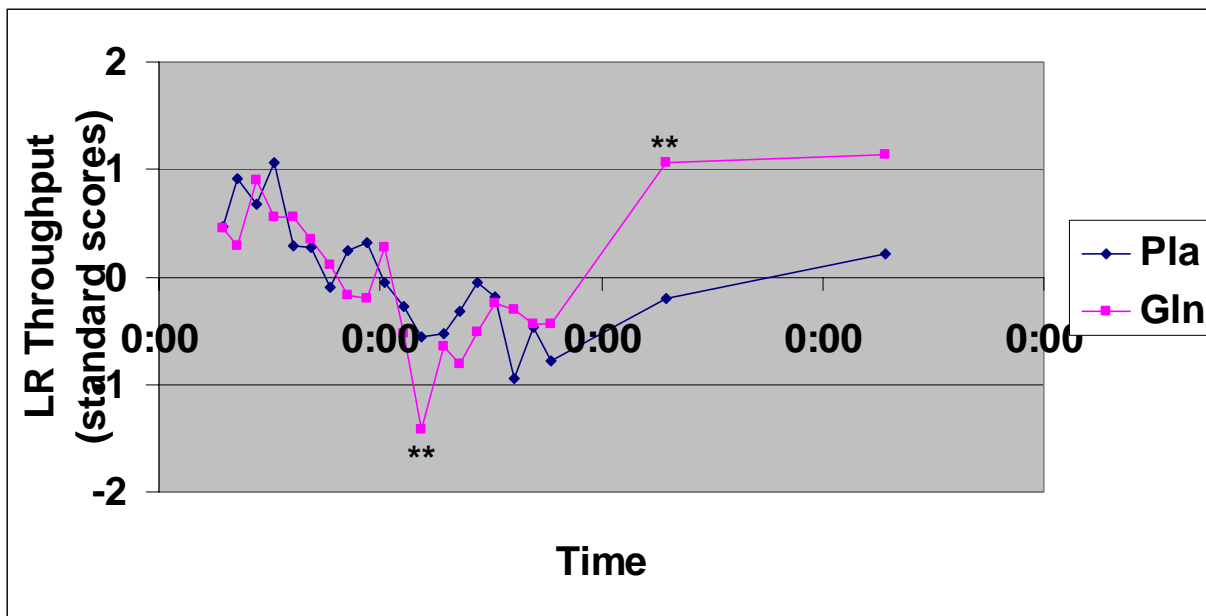


Figure 28: 4-day plot of Logical Reasoning throughput scores (+/-1 SD). Pla Group >Gln Group at 04:30 and 08:30 on Day 2, by multiple t tests (* $p<0.10$).



04.30

Figure 29: 4-day plot of Logical Reasoning throughput standard_scores. Statistically significant differences, by multiple t tests, at 04:30 on the night of sleep deprivation and on the morning of Day 3 (**p<0.05).

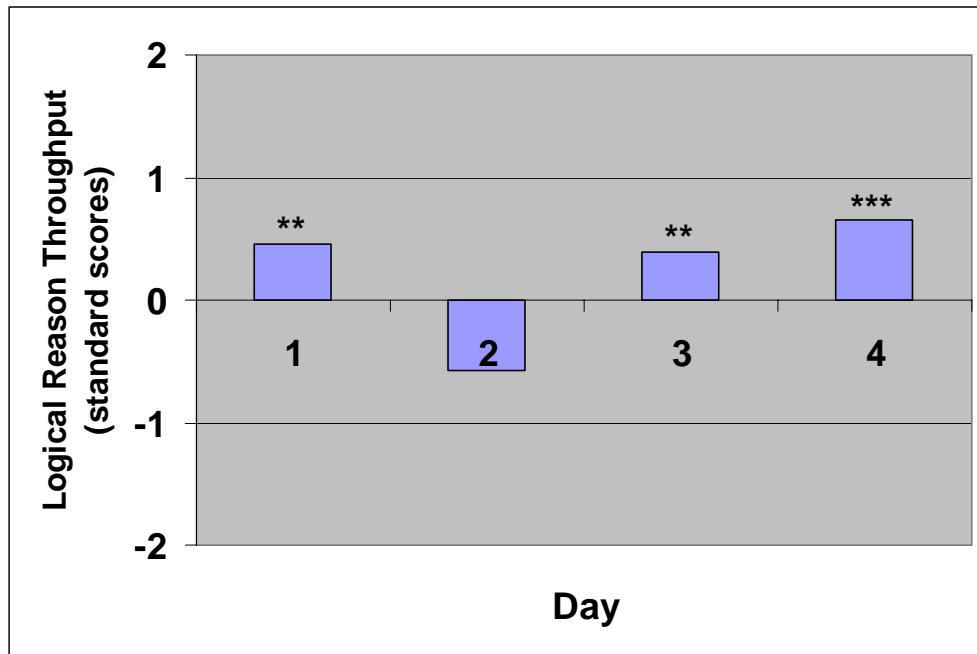


Figure 30: The effect of Day on Logical Reasoning throughput standard score: throughput was significantly higher on Days 1, 3 and 4 than on Day 2 (**p<0.05, ***p<0.01).

Psychomotor-Vigilance Performance

The night of sleep deprivation had an obvious effect on numbers of PVT lapses (Fig. 31). There were no statistically significant differences between groups ($p(t) > 0.10$; tested using the square root transform of lapses to normalize the expected Poisson distribution of lapses).

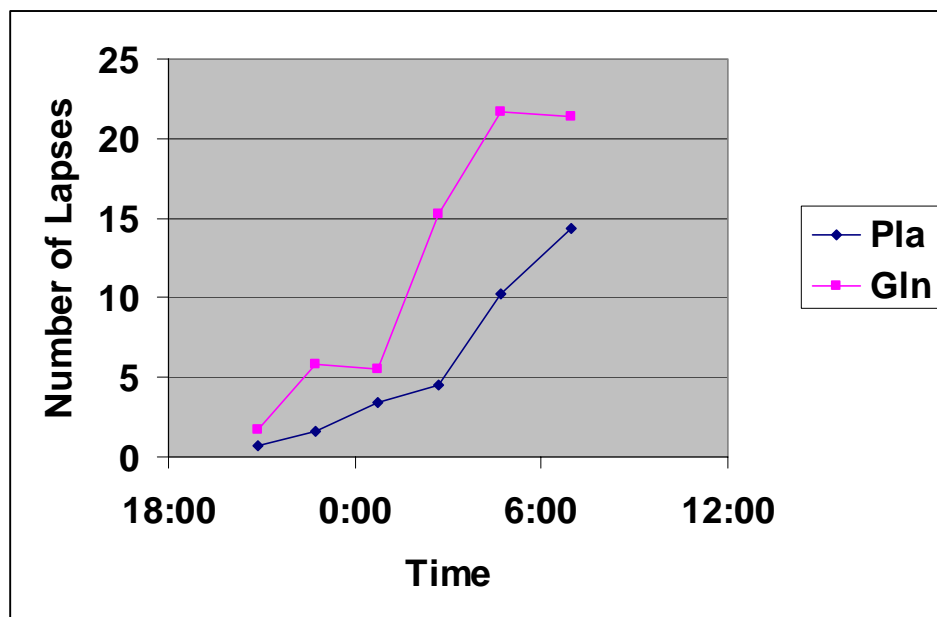


Figure 31: Overnight plot of numbers of PVT lapses (raw numbers). No statistically significant differences between Groups, by multiple t tests on the square root transform of lapses.

The night of sleep deprivation had an obvious effect on mean PVT response speed (Fig. 32). Again, there were no significant differences between Groups ($p(t) > 0.10$). The overnight patterns and inter-group relationships were quite similar for the SRRT and FRRT variables.

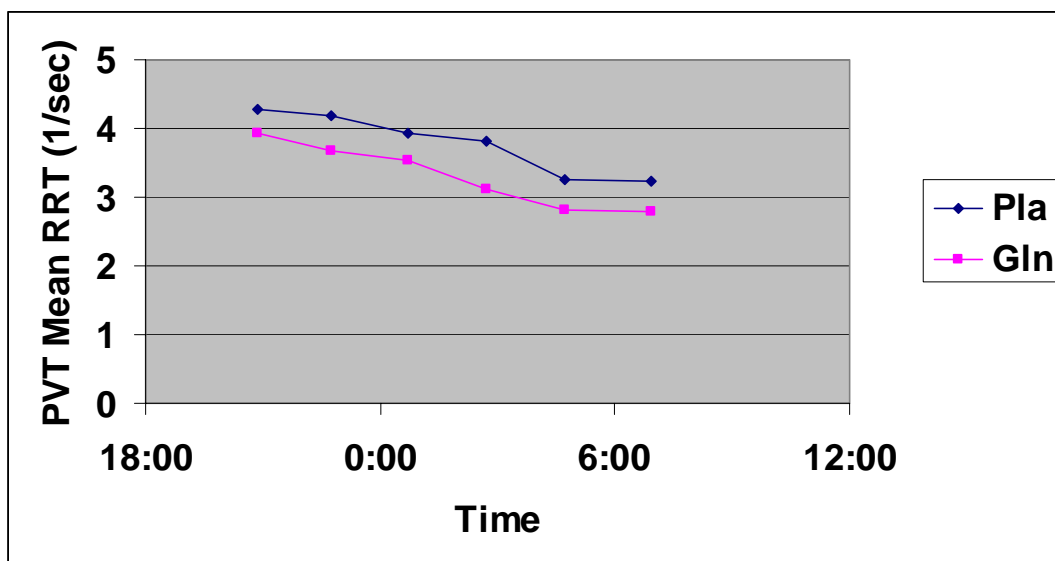


Figure 32: Overnight plot of PVT mean RRT (response speed). No statistically significant differences between Groups, by multiple t tests.

The night of sleep deprivation also had an obvious effect on the slope of the PVT response speed within the 10-minute test period (Fig. 33). Again, there were no significant differences between Groups ($p > 0.10$).

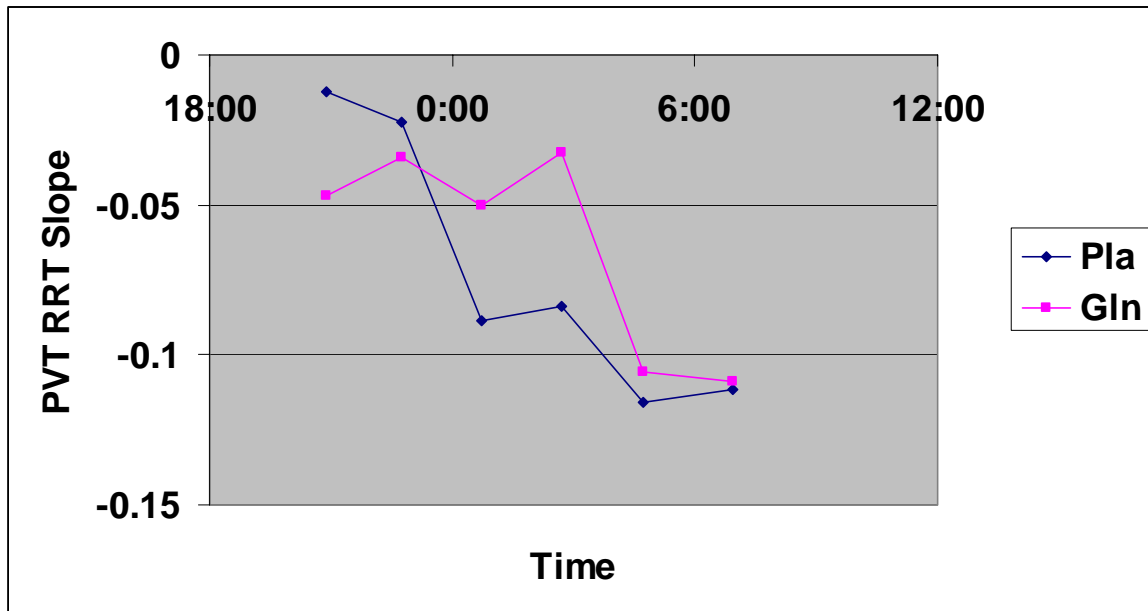


Figure 33: Overnight plot of PVT RRT (response speed) slope. No statistically significant differences between Groups, by multiple t tests.

Two subjects each in the Pla and Gln Groups provided no PVT data on the fourth morning. Thus, we limited our analysis for the effects of the Day factor to the first three mornings of the study for the PVT variables. For purposes of comparison, the Day 2 points in the next three charts are identical to the last points in the PVT overnight charts, above.

There was a significant effect of Day on the square-root transformed lapse count ($F(2,26) = 20.87$, $MSe = 6.82$, $p(GG) = 0.0003$). There was not a significant effect of Group or a significant Group x Day interaction. The *post hoc* assessment of the Day effect indicated that the number of lapses was significantly higher on the morning of Day 2 (immediately following the night of sleep deprivation) than the mornings of Day 1 and Day 3 ($p < 0.01$; Fig. 34).

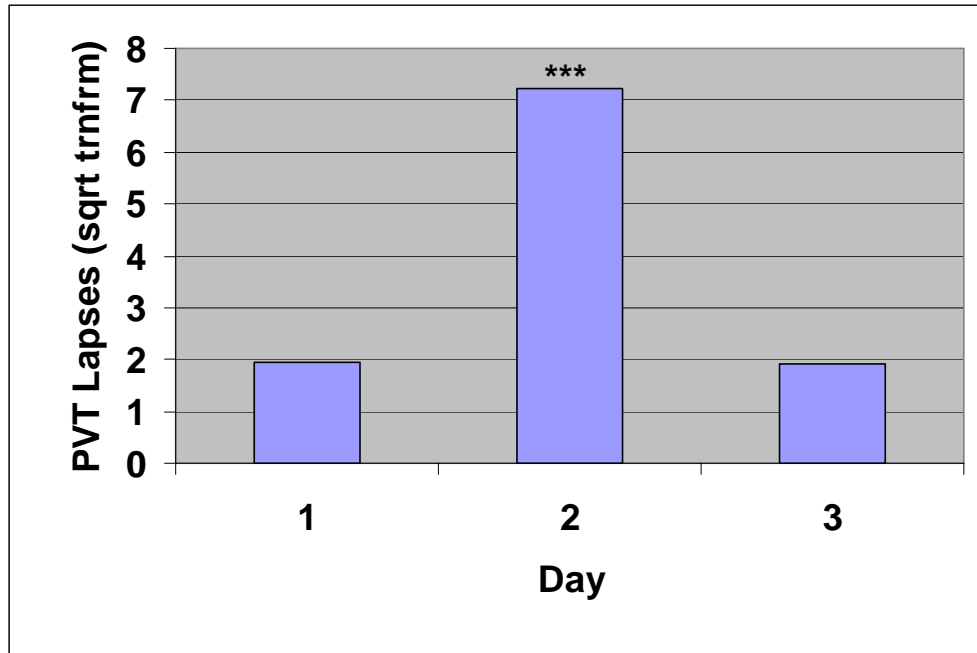


Figure 34: Effect of Day on the number of PVT lapses (square root transform): Significantly higher on the morning of Day 2 than the mornings of Day 1 and Day 3 (***) $p < 0.01$).

There was a significant effect of Day on the RRT (response speed) measures. There were no significant effects of Group or a significant Group x Day interactions for the RRT measures.

The significant Day effects were:

- Mean RRT: ($F(2,26) = 49.91$, $MSe = 0.161$, $p(GG) = 0.0000$)
- Mean fastest RRT: ($F(2,26) = 22.69$, $MSe = 0.115$, $p(GG) = 0.0001$)
- Mean slowest RRT: ($F(2,26) = 55.76$, $MSe = 0.246$, $p(GG) = 0.0000$)

The *post hoc* assessments indicated that response speed was significantly slower on Day 2 than on Days 1 and 3 for all three measures of response speed ($p < 0.01$; Fig. 35).

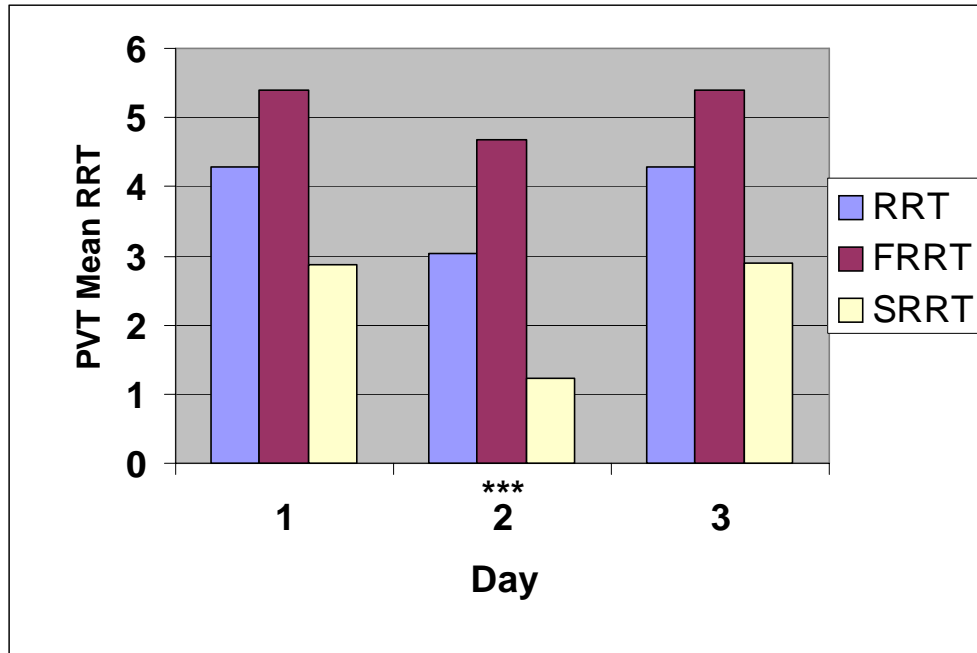


Figure 35: Effect of Day on PVT response speed (grand mean, mean of fastest 10% and mean of slowest 10%): significantly slower on the morning of Day 2 than the mornings of Day 1 and Day 3 for all three measures (*** $p < 0.01$).

There was a significant effect of Day on the within-trial RRT slope ($F(2,26) = 9.68$, $MSe = 0.00334$, $p(GG) = 0.0057$). There was not a significant effect of Group or a significant Group x Day interaction. The *post hoc* assessment of the Day effect indicated that the RRT slope was significantly more negative on the morning of Day 2 than the mornings of Days 1 and 3 ($p < 0.01$; Fig. 36). Thus, response speed declined faster within a 10-min trial on Day 2, suggesting a more rapid loss of the ability to sustain attention.

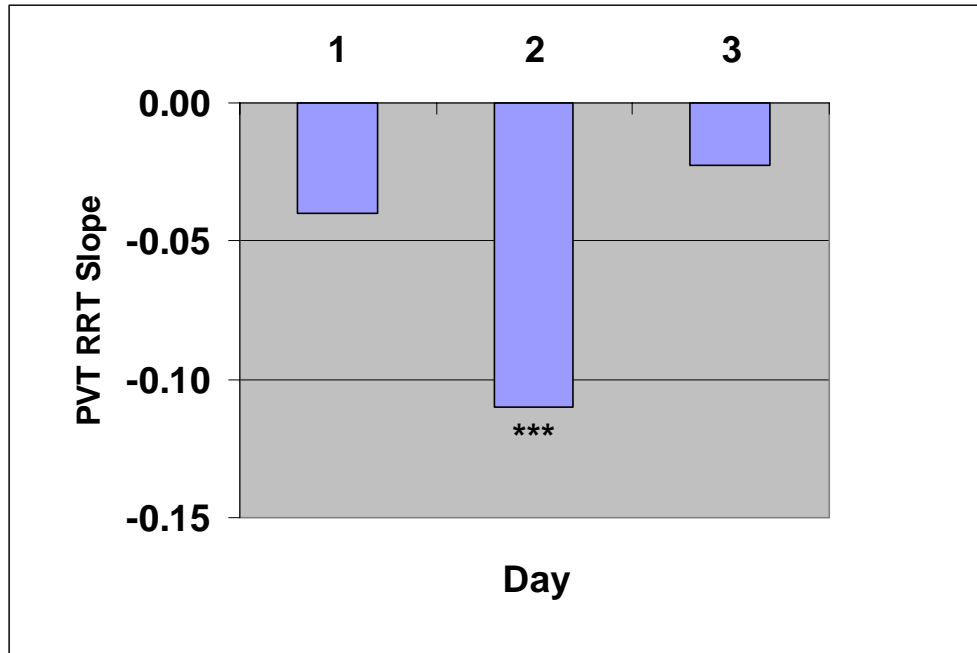


Figure 36: Effect of Day on the slope of response speed during the 10-minute test: Significantly more negative slope on the morning of Day 2 than the mornings of Day 1 and Day 3 (** $p < 0.01$).

Vertical Jump

The three measures available were explosive jump power, jump height, and time spent on the ground between jumps. The 2-factor ANOVA was applied to these three measures as both their within-Day means and their within-Day variabilities (as standard deviation). The analyses revealed only one significant main effect and no significant interactive effects: the effect of Day was statistically significant for jump height ($F(3,24) = 6.53$, $MSe = 0.527$, $p = 0.099$; Fig. 37). The *post hoc* assessment indicated that jump height was significantly lower (about 7.6%) on Day 2 ($p < 0.05$) and Day 4 ($p < 0.01$) than on Day 1.

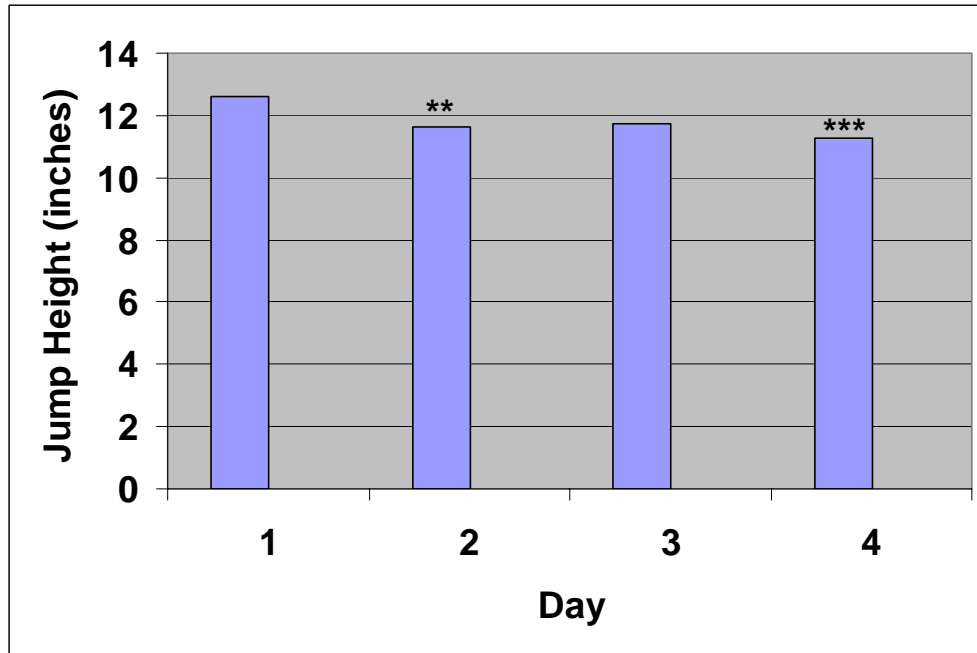


Figure 37: The effect of Day on jump height: significantly lower (7.6%) on Days 2 and 4 than on Day 1 (*** $p < 0.01$, ** $p < 0.05$).

SUBJECTIVE MEASURES

Stanford Sleepiness Scale

Perceived sleepiness increased during the night of sleep deprivation and presented some evidence of cumulative fatigue throughout the next day. The perceived sleepiness of the Pla Group was obviously greater than that of the Gln Group at 18:30 on Day 1 and at 14:30 on Day 2 (2 and 1.5 rating units, respectively). However, neither of these differences was statistically significant when tested using the 2-tail Kolmogorov-Smirnov two-sample test ($p > 0.10$; Fig. 38).

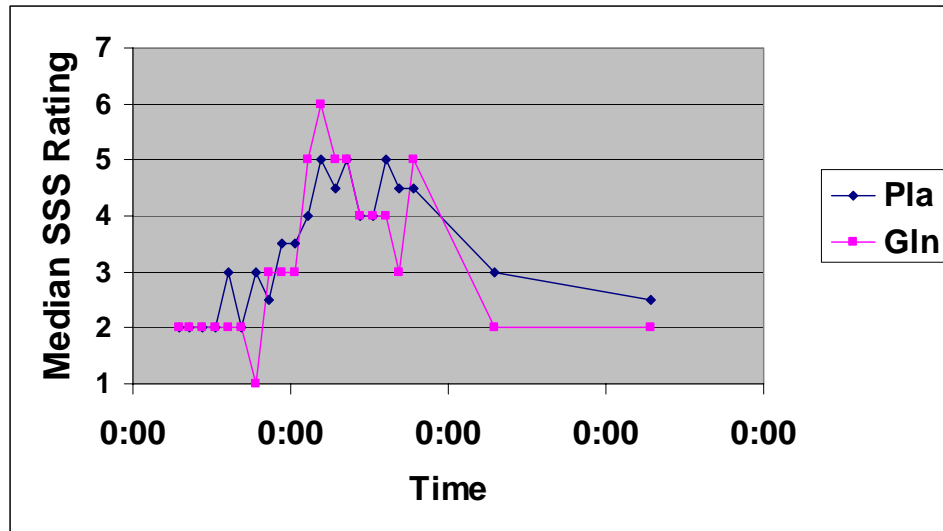


Figure x. 4-day plot of median Stanford Sleepiness Scale ratings.

Mood

The mean Vigour-Activity (VA) and Fatigue-Inertia (FI) factor scores displayed fatigue development throughout one night's sleep deprivation and cumulative fatigue throughout Day 2 (Fig. 39). Recovery was apparent on the mornings of Days 3 and 4. There were no statistically significant differences between Groups at any of these times for either factor ($p > 0.10$).

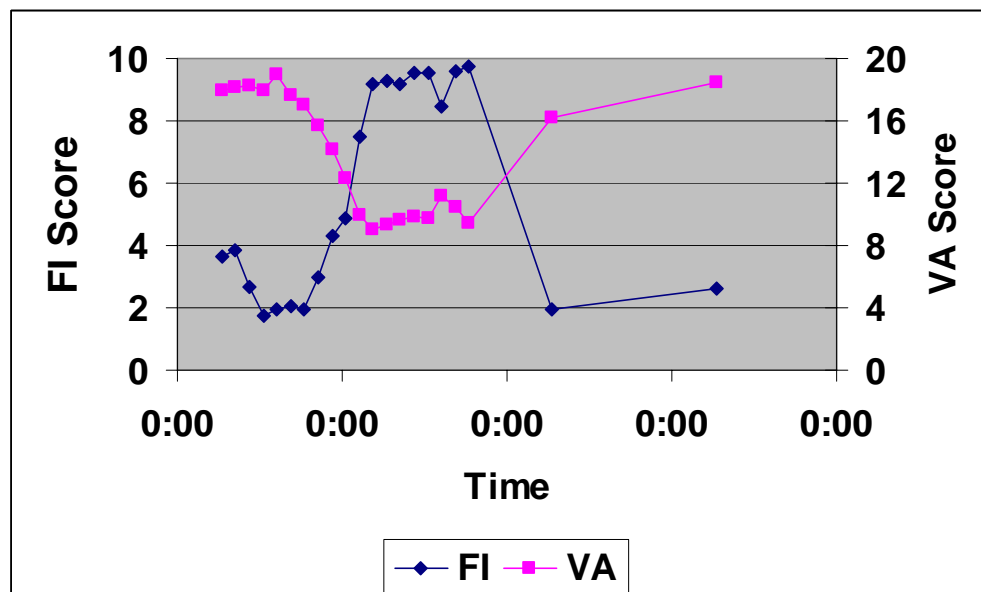


Figure 39: 4-day plot of mean Vigour-Activity (VA) and Fatigue-Inertia (FI) factor scores

When subjected to the 2-factor, 2 x 4-level ANOVA, both the F-I and the V-A factor scores revealed statistically significant effects of Day and no effect of Group or Group-Day interactions ($F(3,39) = 10.55$, $MSe = 16.14$, $p(GG) = 0.0002$; and $F(3,39) = 13.58$, $MSe = 20.34$, $p(GG) < 0.0001$; respectively). *Post hoc* tests revealed that the Fatigue-Inertia score was significantly higher on Day 2 than on Days 1, 3 and 4, and that the Vigour-Activity score was significantly lower on Day 2 than on Days 1, 3 and 4 (** $p < 0.01$; Fig. 40).

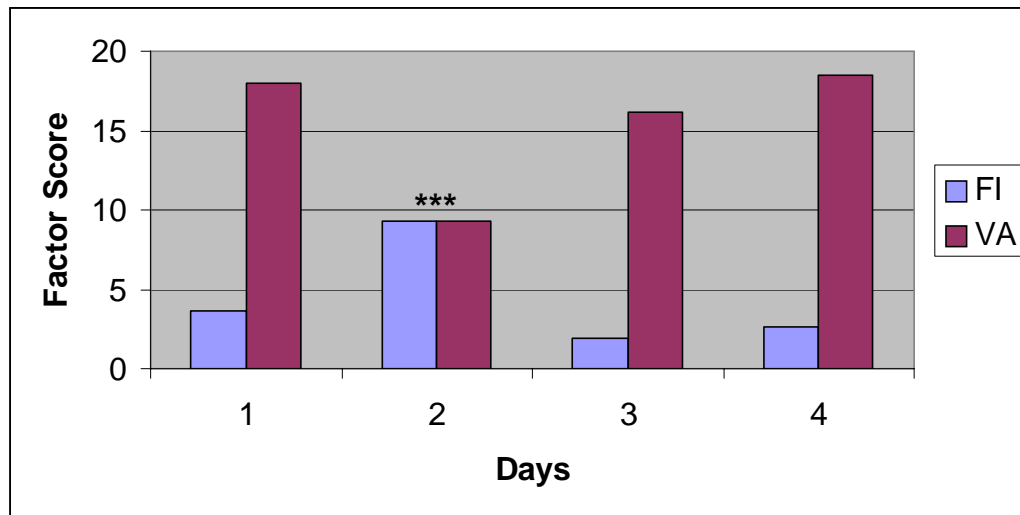


Figure 40: The effect of Day on POMS factor scores: Fatigue-Inertia significantly higher on Day 2 than on Days 1, 3 and 4, and Vigour-Activity significantly lower on Day 2 than on Days 1, 3 and 4 (** $p < 0.01$).

Correlations among Subjective Ratings

The three subjective ratings were intercorrelated as shown in Table 4 ($n = 15$, with 16 observations per subject).

Table 4: Intercorrelations among the three subjective ratings ($n = 15$, 240 observations; FI, Fatigue-Inertia factor score, VA, Vigour-Activity factor score, SSS, Stanford Sleepiness Scale).

	FI	VA
SSS	0.755	-0.641
FI		-0.646

DISCUSSION

An inflammatory response and an effect of one night's sleep deprivation on the immune system was indicated by the significantly increased neutrophil numbers and WBC count on Day 2. This is consistent with reports of lower cell counts during normal sleep and the findings of previous, more chronic, sleep deprivation studies (Born et al., 1997; Dinges et al., 1994). It appears that one night's sleep deprivation elevated the platelet count on subsequent days in the Placebo Group, but not in the Glutamine Group. It is possible that this is linked with the neurotransmitter, 5-HT, which is involved in sleep and fatigue. The plasma concentration of 5-HT is dependent on whether or not the plasma is platelet rich or platelet poor.

Interestingly, patients with obstructive sleep apnoea (OSAS) have increased percentages of activated platelets. Shimizu et al. (2002) also observed that, in nearly 60% of patients, platelet activation remained high despite significant improvement of sleep apnoeic episodes after therapeutic intervention.

NK cells are normally increased as part of the acute phase response to stress. For example, in marathon runners there is marked increase in circulating NK cell numbers after a race, which is maintained for at least 24 hrs. It was therefore interesting to note a rather late increase in NK cell numbers in the Pla group on Days 3 (marked) and to a lesser extent on Day 4. There was no such response in the Gln Group.

Comparing the present study with other studies, sources of variation include the protocol for the night of sleep deprivation. In this study subjects remained alert and active throughout the night of sleep deprivation by regularly completing cognitive and psychomotor vigilance tasks, and completing questionnaires. In contrast, Born et al. (1997) had subjects remain in bed reading, watching television or talking to the experimenter. Thus the combined effect of sustained wakefulness and mental stress may have impacted differently on some immune parameters, such as cytokines, measured in the present study.

Demographics

Data from the Sleep Behaviour Questionnaire indicated that the subjects were typical of other Americans: slightly, chronically sleep deprived and most susceptible to performance decrements in the pre-dawn hours. The inter-Group differences in reported sleep length for nights off and ideal sleep lengths suggested that, compared to their ideal sleep amounts, the

Glutamine Group may have slept less on work nights, chronically, than the Placebo Group and thus required more recovery sleep on weekends. The two Groups were essentially identical with respect to morningness-eveningness tendencies and sleep hygiene knowledge. The degree of depression reported by the two Groups was about the same, with two subjects in the Placebo Group reporting moderate depression and one in the Glutamine Group reporting a clinical level of depression. The two Groups were normal and did not differ in trait anxiety, cognitive hardiness, daytime sleepiness, and circadian acrophase time.

Performance

The two Groups differed at no time in simple response time, PVT lapses or PVT response speed, suggesting a lack of glutamine supplementation effect on fatigue-induced changes in responses to very simple demands on cognitive function. There was a negligible overall effect of overnight wakefulness on simple response time. This is not an unusual finding. There was an expected, marked effect of overnight wakefulness on PVT lapses and response speed.

The Pla Group's absolute performance on the Mental Arithmetic and Logical Reasoning tasks was significantly greater than that of the Gln Group. Standard-score transforms of the data allowed us to see the expected, marked effects of overnight wakefulness: substantial relative decreases in throughput during the pre-dawn hours. It also allowed us to see that the Gln Group had a relative performance advantage of about 14% on both tasks. Scatterplots (Figures 25 and 29) indicated that much of this relative advantage occurred on the mornings of Day 3 and Day 4, after glutamine ingestion began, though the Group x Day interactions were not significant for either task.

Subjective Reports

The two Groups did not differ in reports of sleepiness, Vigour-Activity and Fatigue-Inertia, but did display the expected effects from overnight wakefulness.

***In vitro* cell cytokine production**

Based on the experiences of the pilot study it was decided to measure *in vitro* cell production of cytokines rather than plasma cytokine concentration, since some cytokines of interest proved to difficult to measure in blood samples taken at rest. IL-1 was not detectable at

all; IL-6 and IL-8 were only detectable in those with cold/fever. Using a different approach to preparation, the incubation medium used to dilute whole blood samples prior to incubation did not have added glutamine (or foetal calf serum). This means that the *in vitro* glutamine concentration reflected the individual's p[Gln] at the time of the blood sample being taken. This technique has been used routinely by the PI, who considers it preferable to putting cells into an "unnatural", though optimum, concentration of 2mM glutamine as is commonly done for isolated cells. The mitogenic stimulant, Concanavalin A (Con A) was used in the present study.

Yaqoob & Calder (1998) reported small increases in IL-1 α , IL-1 β , TNF- α and IL-6 production from Concanavalin A (Con A)-stimulated peripheral blood mononucleocytes (PBMN) cells (monocytes and lymphocytes) in the presence of 0.1mM Gln. However, Rhode et al. (1996) found no effect on IL-1 β , TNF- α and IL-6 production by phytohaemagglutinin (PHA)-stimulated human blood mononuclear cells in the presence of 0.6mM Gln. This may be due to the fact that the latter study used a mitogen (PHA) which was not specific for CD3 cells (T lymphocytes) as is Con A.

IL-2 and IL-3 production

In the present study there was a non-significant trend towards a decrease in IL-2 production on Day 2. Previous studies have demonstrated that partial and total night's sleep deprivation significantly decreased stimulated IL-2 production (Born et al., 1997; Irwin et al., 1996). IL-2 production is normally reduced with the stress of prolonged, exhaustive exercise (Castell, 2003).

Due to high individual variation, there was no significant effect of either Day or Group on IL-3 production. There was no similarity of pattern with the other growth factor measured, GM-CSF.

IL-6 production

One night's sleep deprivation did not appear to affect IL-6 production by T lymphocytes in the Pla Group. There was a significant decrease on Day 2 in the Gln Group compared with the Pla Group. One night of recovery sleep was sufficient to restore T lymphocyte IL-6 production to baseline values. However, WBC numbers remained significantly higher on Day 3 compared to baseline. Increased relative amounts of slow wave sleep (SWS) is typical of

recovery sleep and there is evidence of SWS being negatively correlated with p[IL-6] (see Irwin, 2002). This contradicts the results of this study where IL-6 production from T lymphocytes increased after a night of recovery sleep. However, the source of IL-6 in sleep has not been documented. Therefore, it is possible that, although IL-6 production by T lymphocytes increased, this may not have been the case for all sources of IL-6.

The wide intersubject variation in IL-6 production may in part be explained by diet and body fat. There have been no studies on the effect of fatty acids (FA) on IL-6 production. However, IL-1 is affected by dietary FA: since IL-1 can induce IL-6, the possibility that diet could affect IL-6 production cannot be ruled out. When the subjects remained in the laboratory on Days 1 and 2, the diet was standard for all subjects, however between breakfasts on Days 3 and 4 there were no restrictions on diet. A more likely cause for variation in IL-6 production is body fat mass. Subcutaneous adipose tissue secretion of IL-6 has been demonstrated to correlate with body mass index (BMI) (Mohammed-Ali et al., 1997), as has serum IL-6 and BMI (Bastard et al., 2000). Although this suggests adipose tissue contributes to high circulating IL-6, other sources have not been investigated in such situations.

In the Pla group six out of eight individuals had decreased IL-6 production on Day 2, but the remaining two subjects had 5- and 8- fold increases. It is questionable as to whether the effect of sleep deprivation on IL-6 production has large intersubject variability. The IL-6 data from these two subjects (both of whom reported illness during the study) may be representative of an immune response to a challenge present before the night of sleep deprivation, or to a T cell response to acute sleep deprivation.

Hypotheses for this study were that one night's sleep deprivation would 1) cause sufficient stress to increase production of proinflammatory cytokines as in the early immune response and 2) impair immunocompetence leading to increased risk of illness. Thus these two subjects cannot be eliminated from the analysis as they represent results that would be expected in this situation. It would have been helpful to have had more subjects enrolled in the study.

Decreased IL-6 production by T lymphocytes can be used to indicate the predominant type of T helper (Th) immunity in these circumstances. Th1 cells secrete IL-2, IL-12 and IFN- γ , and Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13. Furthermore, cytokines secreted by each subset act to inhibit development of the opposing subset (see Smith, 2003). It is possible that a decrease in IL-6 production may indicate the dominance of a Th1 response the morning

after sleep deprivation. This is supported by a concurrent increase in neutrophils and WBC count. Studies on the effect of sleep deprivation on immunization support this idea. It has been observed that a normal night's sleep after vaccination against hepatitis A and influenza improves humoral immunity, characterised by increased antibody production, compared to that observed when total sleep deprivation or restricted sleep ensues (Lange et al., 2003; Spiegel et al., 2002). However, it seems that when an immune challenge is present during the period of sleep deprivation, as with the two subjects in the PLA group, acute sleep deprivation does not impair the Th2 response, which has no coherence with the findings of the immunization studies. One reason for this may be that in the immunization studies subjects had not previously been exposed to the antigen, which they were immunized against.

The onset of sleep has been associated with increased circulating levels of IL-6 (Vgontzas et al., 1999, 2003): when the onset of sleep is delayed until the second half of the night (03:00) so is the increase in IL-6 (Redwine et al., 2002). Thus it is possible that when sleep is delayed even longer, so is the main, sustained increase in circulating IL-6. There are no studies which have documented p[IL-6] or IL-6 production throughout a night of sleep deprivation. The cellular source of IL-6 variations in sleep has not been established.

IL-8 production

This is the first time that IL-8 production has been measured after a night of sleep deprivation. Each group displayed a distinctly different pattern for IL-8 production. There was a close to significance trend for the groups to be different on Day 1, with IL-8 production being higher in the Pla Group. After exclusion of subject 290 (for the same reasons as for IL-6) there were close to significance differences on Days 1 and 4.

In the Pla Group, IL-8 production tended to decrease on Day 2, the morning after sleep deprivation, and this was more marked with the removal of subject 290. Previous studies have reported a relationship between sleep deprivation and increased leukocytosis (Born et al., 1997; Dinges et al., 1994), which was replicated in this study. The increased neutrophilia observed in this study the morning after sleep deprivation (Fig. 7) may not have been related to T cell IL-8 production in the Pla Group. Neutrophils are the primary producer of their own chemoattractant and the contribution of T cell-derived IL-8 in this process is poorly documented. Additionally,

IL-8 is produced by a variety of cells; thus it cannot be assumed that acute sleep deprivation affected IL-8 production similarly in all cell types.

It is tempting to speculate that, as with IL-6, sleep deprivation somehow impairs IL-8 production by T lymphocytes. However, subject 290 who reports illness on Day 3, had distinctly increased IL-8 production on Day 2; thus it seems more likely that, in the absence of an appropriate stimulus, IL-8 production from T cells at least is not affected by sleep deprivation.

If IL-8 production in sleep and during sleep deprivation, is dependent on circadian rhythm then a single morning blood sample may have not been sufficient to detect any changes. One night of recovery sleep did not restore the decrease in IL-8 production to baseline values, even with subject 290 excluded. With the exclusion of subject 290, after two nights of recovery sleep, IL-8 production increased and reached near baseline levels. As recovery sleep is characterised by increased amounts of slowwave sleep (SWS) (see Irwin, 2002) it would be interesting to see, if like IL-6, IL-8 is negatively correlated with amounts of SWS.

There was a wide individual variation in IL-8 for Day 1 in the Pla Group. Studies concerning IL-8, fat mass and diet, which could in part explain this variation, are limited. p[IL-8] is higher compared to controls in obese subjects (Bruun et al., 2003), but it is only correlated with BMI when BMI~ 20-30kg/m (Bruun et al., 2002).

GM-CSF production

Overall, there was a close to significance decrease in GM-CSF on Day 2 compared with Day 1: the level on Day 2 was significantly ($p<0.05$) lower than that on Day 3. The overall data followed a similar pattern for both Gln and Pla Groups, and returned to baseline levels after a night's sleep recovery. This may be indicative of transient immunodepression due to one night's sleep deprivation. It has been proposed that GM-CSF has a direct effect on sleep regulation through the CNS (Kimura et al., 1999). Since GM-CSF and its receptors are expressed in the brain it was suggested that GM-CSF may affect various functions of the central nervous system (CNS). In a study on rats GM-CSF significantly increased both non-rapid eye movement and rapid eye movement (REM) sleep, or REM sleep only, when infused intracerebroventricularly. Findings of a subsequent study (Kimura et al., 2000) indicated that centrally administered GM-CSF stimulates the release of somatostatin (a hormone which promotes REM sleep) via activation of the nitric oxide system in the hypothalamus.

Glutamine Supplementation and Cytokines

There was a non-significant trend for IL-8 production to increase the morning after sleep deprivation and return to baseline after a night of recovery sleep in the Gln Group. This is the first study in which oral Gln feeding has been related to increased IL-8 production. In all previous GLN feeding studies, both clinical and exercise related, a concurrent decrease in IL-8 production has been reported (see Castell, 2003 for a review). The rationale behind this was based on the assumption that human neutrophils use Gln, whereas this had only previously been demonstrated in rat neutrophils (Curi et al., 1997). This argument was strengthened by Castell et al. (2004) who recently reported the presence of glutaminase on the surface of human neutrophils. It was thought that the supply of exogenous Gln satisfied neutrophil demands leading to a reduced need for further chemical signalling, via release of the chemokine IL-8, to attract more neutrophils (see Castell, 2003). An *in vitro* study by Coëffier et al. (2001) observed decreased IL-8 concentrations in human duodenal tissue cultured with 2mM of Gln compared to control.

Despite this, the presence of more neutrophils is an advantage to host defence and an increase in IL-8 production in the Gln Group could be evidence of an increased T cell mitogenic response. However, the decrease in p[Gln] the morning after sleep deprivation was small (34µmol). A marked p[Gln] decrease is observed immediately after prolonged exercise or trauma, partly because increased amounts of Gln are going intracellular and thus removed from the circulation *per se*. It must be noted that, in the present study, resting samples were taken which would not reflect such large changes. Thus after one night's sleep deprivation, and with adequate calories the night before, the decreased p[Gln] is almost certainly a response to stress, possibly due to cell migration in response to a perceived immune challenge. Increased neutrophil numbers the morning after sleep deprivation were observed in both groups. However, it is possible that there were different cell sources for the IL-8 production thought to be essential for this. It has been demonstrated that human monocytes *in vitro* can produce IL-8 in response to complement C5a (Ember et al., 1994).

There was a near significant negative correlation between mean IL-6 and IL-8 in the Gln Group over the four days. This suggests that when Gln is provided, sleep deprivation and recovery sleep have opposing effects on the T cell production of these cytokines. This has not previously been reported in *in vitro* or *in vivo* studies.

However, non significant changes in both groups suggest that one night of sleep deprivation does not affect IL-8 production the next morning. Thus, it can be speculated that IL-8 has a biphasic response to sleep deprivation, where one night of sleep deprivation is not a sufficient stressor to increase amounts produced by T-cells significantly. Additionally, it is also possible that at another time during the day after sleep deprivation the situation was not the same. It would have been useful to have been able to take additional blood samples at other time points.

There was no effect of glutamine on IL-2, in which both Groups followed the same pattern, or on IL-3 production.

Leptin

One night of sleep deprivation did not have a significant effect on p[Lep] in either group and p[Lep] did not change significantly over the four days. This is consistent with the findings of Mullington et al. (2003) who reported no significant differences in serum leptin after one, two and three nights of sleep deprivation compared to baseline. However, in the pilot study prior to the present project, eight out of twelve subjects had a 24% decrease in p[Lep] on Day 2 (Barton et al., 2003). This is a similar decrease to that observed in subjects whose sleep duration was reduced by 6hrs per night for several nights in a study by Spiegel et al. (2004), with slightly less of a decrease after 4hrs sleep loss. These authors found that decreases were quantitatively associated with alterations of the cortisol and thyroid stimulating hormone (TSH) profiles. The impact of stress on leptin appears to depend on the type, intensity and duration of the stress. The more prolonged and/or higher intensity the stress, the greater the decrease in p[Lep]. Ricci et al. (2000) found decreased p[Lep] during cold exposure – it is interesting to speculate that the normal decrease in oral temperature which was observed during the present study might be linked to this, which was held to be due to activation of the systemic nervous system.

Circulating leptin levels display a diurnal variation with highest concentrations observed at midnight and lowest concentrations around noon (Kanabrocki et al., 2001; Sinha et al., 1996), but a circadian rhythm for leptin is disputed (Schoeller et al., 1997). Oral temperatures indicate there was no change in the phase of circadian rhythm over one night of sleep loss, however the work of Mullington et al. (2003) indicated that serum leptin is also under the influence of sleep. Thus, despite following the same pattern of elevation during the night, sustained wakefulness

(even one night) reduced the amplitude of the peak, and this returned to normal in the first night of recovery sleep. Even though morning p[Lep] remained the same the morning after sleep deprivation, this does not necessarily reflect an unaltered p[Lep] throughout the night. This is at variance with the findings of Schoeller et al. (1997) who reported one night of sleep deprivation to have no effect on the diurnal amplitude of leptin. It has not been considered in the literature, but it is possible that the enhancement of circulating leptin during sleep is associated with its restorative function and it may possibly interact with some components of the immune system to achieve this.

Implications of changes in p[Lep] for Immune Function

Although there were no significant effects of one night of sleep deprivation on p[Lep], it is interesting to note that six out of fifteen subjects had decreased p[Lep] the next morning (Day 2). Circumstances associated with decreased leptin levels, such as starvation, or where leptin does not function properly, such as in obesity, also relate to immunodepression (Howard et al., 1999; Lord et al., 1998). Reduced circulating CD4⁺ T cells, impaired T cell proliferation and cytokine release in leptin deficient children and children with moderate protein calorie malnutrition are attributed to the reduced circulating leptin levels observed in these conditions.

The leptin receptor is expressed in human PMN (Caldefie-Chez et al., 2001), peripheral blood monocytes (Najib & Sánchez-Margalet, 2002) and T lymphocytes (Martin-Romero et al., 2000). Thus it is thought that leptin can influence these cells and enhance the proinflammatory response; this is supported by *in vitro* studies. In *in vitro* studies, leptin has the capacity to intensify the primary T cells response, to increase peripheral blood CD4⁺ proliferation after antigen recognition, and to increase IL-6 production by monocytes, and IL-2 and IFN- γ production from stimulated T lymphocytes (Lord et al., 1998; Martin-Romero et al., 2000; Santos-Alvarez et al., 1999).

Diet and Leptin

There are several explanations for the considerable variation in leptin results noted in this study. First is the relationship between p[Lep] and body fat mass. Several studies using athletes and normal subjects have noted p[Lep] to be highly correlated with fat mass (Hickey et al., 1996; Ronnema et al., 1997). Secondly, there are the effects of energy availability and food intake on

leptin levels. Energy availability affects circulating leptin concentrations: as little as 24 hrs fasting decreases mean leptin by 40% compared to baseline, and after 72 hrs circadian variations are eradicated (Wagner et al, 2000). After a meal, serum leptin progressively increases for up to eight hours, depending on the nutritional content of the meal, and decreases for similar lengths of time after the onset of fasting. This is thought to be independent of circadian rhythm (Dalloneville et al., 1998). The effect of meal time on p[Lep] has also been considered and evidence points to the diurnal rhythm of p[Lep] being coupled with meal pattern. Schoeller et al. (1997) demonstrated a shift in p[Lep] that corresponded to a shift in meal times, and this shift occurred within one day. In the same study, plasma cortisol was unaffected so it was concluded that p[Lep] does not adhere to a circadian rhythm. Finally, dietary composition must be considered. It has been suggested (Cusin et al., 1998; Nemezc et al., 1999) that leptin may have an important role in switching fuel utilization from carbohydrate to fat during stressful conditions. Studies have yielded various results. During periods of massive overfeeding, serum leptin can reach 40% above baseline values within 12 hrs (Kolaczynski et al., 1996). A series of tests by Herrmann et al. (2001) concluded the pattern and amount of leptin secretion may be influenced by high glycaemic index carbohydrate or the simple sugar content of the diet. Other studies have not observed any effects of high or low fat meals on p[Lep] (Weigle et al., 1997).

So, it is possible that the variation in diet and body fat mass of the subjects in this study affected p[Lep] on Day 1. The subjects ate in the laboratory from the morning of Day 1 until the morning of Day 3; thus it is possible that daily variations in p[Lep] were synchronised across these days. However, body fat mass and diet prior to the study would still affect p[Lep].

There was a near significant correlation between mean p[Lep] and p[Gln] over the four days. The likely reason for this is that plasma levels of both are affected by diet, however, lack of information on the subjects' diets outside the experimental period (due to poor compliance) means that clear conclusions cannot be drawn. There was a significant correlation between mean p[Lep] and p[Caff] in both groups over the four days. This may have been due to caffeine increasing lipolysis and p[Lep] being sensitive to changes in adipose tissue.

Plasma Glutamine Concentration

This is the first study in which p[Gln] has been measured after sleep deprivation in humans. On Day 2, the morning after sleep deprivation, p[Gln] was maintained in the Gln Group

and there was a non significant 5% decrease in the Pla Group. The groups did not differ significantly from each other on this day, mainly due to the high SEM for the Gln Group caused by a wide range of values. The non-significant 5% decrease suggests that, although small, sleep deprivation affected p[Gln], and that this could be attenuated with Gln supplementation.

It is questionable as to whether this non-significant 5% (34 μ mol) decrease the morning after sleep deprivation was substantial enough to affect immune function in the Pla Group. Lymphocytes cannot synthesise Gln as they lack the enzyme glutamine synthetase and therefore depend upon blood Gln to meet their metabolic requirements (Ardawi & Newsholme, 1982). The mean p[Gln] for either group did not deviate from the normal physiological range of 500-700 μ M on any morning of the study. Previous studies reporting that decreased p[Gln] correlated with immunodepression have observed greater decreases in p[Gln] than in this study. Parry-Billings et al. (1990) observed, in *in vitro* studies, that decreasing Gln concentration in cell culture medium from 0.6 to 0.05 mmol/L considerably decreased human lymphocyte proliferation in response to the mitogen Con A, and slowed the response time.

It appears that a night of sleep deprivation and two nights of recovery sleep caused a decrease in p[Gln] in the Pla Group; it seems that Gln supplementation modified this decrease. On Day 4, there was a close to significance trend for p[Gln] to be on average 105 μ mol lower in the Pla Group and 51 μ mol lower in the Gln Group compared to Day 3. The reason for this is unknown, but it would have been useful to have monitored levels on the evening of Day 3. The degree of decrease evident in the Pla Group after sleep deprivation on Day 4 is closer to that observed after exercise than in clinical studies and may contribute to compromised immune function. Again, it must be emphasised that these are resting samples and not post-exercise. Parry-Billings et al. (1992) and Castell et al. (1996) reported p[Gln] to decrease by 20-25% (100-150 μ mol) after a marathon. This decrease is concomitant with both the leukocytosis and the decreased numbers of circulating lymphocytes observed in the recovery period (Castell & Newsholme, 1997). When a Gln drink was provided post-marathon, numbers of leukocytes and neutrophils were restored to baseline the following morning compared to controls (see Castell, 2003), and the incidence of illness was decreased by 40% (Castell & Newsholme, 1997). Similar results, together with an increase in cytokines, have been observed by Bassitt et al. (2000) after provision of a Gln precursor.

It has not yet been established what is the precise reason for this, and which aspects of the immune system are affected by Gln. Several studies have shown that provision of Gln does not appear to have a positive effect on some aspects of the immune system impaired by exercise despite p[Gln] being maintained. Salivary IgA, pattern and numbers of lymphocytes, monocytes and neutrophils at comparable time points, and lymphocyte proliferation have been demonstrated to be similar in subjects receiving Pla and Gln supplementation after prolonged, high intensity cycling (Krzywkowski et al., 2001; Rohde, MacLean & Pedersen, 1998). These and other studies are reviewed in Castell (2003). The observed decrease in p[Gln] on Day 4 in this study in the Pla Group may not have necessarily have impaired immune function compared to the Gln Group.

The effects of Gln supplementation compared to Pla on the immune parameters measured are not clear; IL-6 and IL-8 have been discussed previously. There were no significant differences between the Pla and Gln Groups in WBC count, neutrophil numbers or CD19+CD3- counts on any of the days. With regard to the increase in circulating neutrophils and WBC numbers on Day 2, it is possible that more Gln was used by these cells, leaving p[Gln] decreased in the Pla Group. In the Pla Group, p[Gln] returned to baseline on Day 3 despite a persistent increase in WBC and neutrophil counts. This suggests that a night of recovery sleep is sufficient to restore p[Gln] to baseline levels without resulting in a p[Gln] deficit in active cells. There was a 46% increase in NK cell numbers in the Pla Group on Day which appeared to be maintained to a lesser extent on Day 4. It is not possible to establish whether this is beneficial, i.e. more NK cells working harder as opposed an inhibitory effect of glutamine in the other group, or whether less cells were required in the Gln Group because they had sufficient Gln to work efficiently. In the earlier prospective study a 2.5-fold increase in basophil numbers in the Gln group was observed. Unfortunately the sensitivity of the WBC assay was poor for the present study, thus no other granulocyte numbers, other than neutrophils were available.

p[Gln] and Cognitive Function

However, the maintenance of p[Gln] does seem important in terms of data for the logical reasoning and mental arithmetic tasks of the ANAM; the Gln Group performed better than the PLA group on this measure on the mornings of Days 3 and 4. Young et al. (1997) observed a mood-enhancing effect in bone marrow patients with the provision of Gln, and it is well

documented that sleep loss affects rate of perceived exertion (RPE), mood and cognitive function. This is the first study to show that Gln can modify some of the effects of sleep deprivation on cognitive function.

Profile of Mood States

The POMS scores were only applied to Fatigue-Inertia and Vigour-Activity. In retrospect it would have been interesting to measure some other parameters such as anxiety, tension, depression, which might have had links with some immune system parameters such as salivary IgA.

Caffeine

Subjects were asked to refrain from consuming caffeine for ten hours before the study commenced and no caffeine containing drinks were available on the days spent in the sleep laboratory (Days 1 and 2). Thereafter caffeine consumption was the subject's choice.

On Day 1 of the study there was a tendency for p[Caff] in the Gln group to be higher than that of the Pla Group. This in part is the result of the contribution of one subject in the Gln Group who had p[Caff] of over 500ug/ml and two other subjects with p[Caff] in excess of 100ug/ml. There was a significant decrease in the Pla Group and a non significant decreased in the Gln Group on Day 2, the morning after sleep deprivation. This is the likely result of caffeine not being available in the daytime of Day 1 of the study when subjects remained in the laboratory. p[Caff] remained low in both groups on Day 3; subjects remained in the laboratory until the morning of Day 3 where caffeine was not available.

On Day 4 there was a non significant trend for a difference in p[Caff] between Pla and Gln Groups, and p[Caff] was increased significantly in the Pla Group on Day 4 from Day 3. The two subjects with the highest p[Caff] in the Pla Group on Day 4 report usual caffeine per day scores of 0.5; suggesting they usually drink less than one cup of coffee per day but after sleep deprivation consume 3-4 cups. Thus it seems that on Day 4 there has been a reversal in p[Caff] in the groups compared to Day 1, suggestive of a restorative effect of Gln supplementation on recovery from sleep deprivation, that is if caffeine intake is presumed to reflect a feeling of sleepiness. This is supported by results of the ANAM in which throughput for the mental arithmetic and logical reasoning tasks was significantly higher (suggesting a better performance)

in the Gln Group on Days 3 and 4 compared to the Pla Group, as well as at the end of Day 2, the day after sleep deprivation. However, this was not reflected in the subjective measures (SSS and POMS); PVT data for Day 4 was not available.

It must be noted that blood samples were drawn resting and fasting on each morning of the study between 06:30 and 07:00. Caffeine levels peak after oral ingestion of cola or coffee within approximately 45 min of ingestion; this occurs later (approximately 67 min) if capsules are ingested, and 99% of caffeine is absorbed (Liguori et al., 1997). Yet one subject in the Pla Group and two in the Gln Group had p[Caff] over 30ug/ml on Day 2 when caffeine had not been available for over 24 hrs.

One subject maintained caffeine levels above 80 g throughout the study, despite no access to caffeine. He was also the oldest subject, had fairly high alcohol consumption, smoked one cigar per month, and admitted to only two cups caffeine per day on a normal basis. However, this is difficult to believe sine his caffeine levels were so high at the start. There was one other subject who was very high at the start of the study.

Implications of caffeine on immune function are rarely considered. Monocytes express the A2 adenosine receptor (Prabhakar et al., 1995), and it is thought that the A2a receptor is the main subtype in lymphocytes (Huang et al., 1997); caffeine is an A2 receptor antagonist. TNF- α release by leukocytes in response to stimulation with *Streptococcus pneumoniae* was inhibited by 200 μ g/ml caffeine (van Furth et al., 1995). Furthermore, the inhibitory effects of IL-12 were reversed by addition of 10^{-6} of caffeine in whole blood *ex vivo* monocytes cultures (Link et al., 2000). The effect of caffeine on immune parameters at physiological concentrations needs further investigation.

Cortisol

There was a non-significant trend towards an increase salivary cortisol on Day 4. Plasma cortisol, on the other hand, was significantly different between the Pla and Gln Group, with the Gln Group having 24% higher cortisol on Day 4 than the placebo group. The data for both these milieu compares favourably with the findings of Leproult et al. (1997), who observed that cortisol was increased the evening after sleep loss. Plasma cortisol concentrations are linked to immune function.

Salivary IgA

The progressive increase in salivary IgA (s[IgA]) from Days 1 to 4 for all subjects combined is interesting. In other conditions of stress such as habitual strenuous exercise (Gleeson 2000), for example, the tendency is for s[IgA] to decrease. It has also been observed to decrease in conditions of anxiety.

Metabonomics

The analysis of this data is complex, despite the use of a new technique known as pattern cluster recognition, which makes it possible to distinguish interesting clusters from the mass of spectra generated by the NMR technique. The noticeable increase in urinary methylhistidine is interesting although difficult to interpret. Urinary methylhistidine is a sign of muscle degradation and is most commonly observed after exercise. In the only sleep study that has looked at urinary methylhistidine levels, Adam et al. (1986) saw no difference in levels between good and poor sleepers. It has not yet been possible to establish whether, as hoped, clear changes are noticeable for leucine, isoleucine, valine and tryptophan is available within these metabolic profiles. The plan was to assess the plasma concentration ratio of free tryptophan to branched chain amino acids in order to investigate the central fatigue hypothesis. As yet, no marked alterations in the plasma profiles have been observed. More detailed analysis may enhance the relevant spectra.

Physical Performance

Vertical jump performance is a standardized measurement for explosive leg power and has been shown to be sensitive to sustained operations. However, previous investigations have included multiple stressors (sleep loss, intense physical work, and hypo caloric diets). This investigation allowed the assessment of the effects of a single stressor: sleep loss. We observed an 8% decrement in jump height between the initial value on the first morning and the morning following sleep deprivation and an 11% decrement in jump height between the initial value and the final value after two subsequent nights of recovery sleep. This has important ramifications for physical/athletic performance.

Limitations to the Study

With hindsight (as happens in many studies) adjustments could have been made to the protocol which are likely to have improved the outcome. First, only one blood sample was taken (fasting, early a.m.) on each of the four days, thus any changes in the blood parameters measured during the night or over the course of the day were not documented. Additionally, the peripheral blood sample is a small representative sample (ca. 1%) of the body's total immune cells. There are multiple sources of the cytokines studied here, and alterations in production by any of them as a result of sleep deprivation may be important.

Given the complexities and cost of keeping subjects in the sleep lab for 2-3 days, it was decided to recruit sixteen subjects, with eight in each group. However, one dropped out just before the study began, and it was impossible to get a blood sample from one subject on the crucial morning, i.e. just after sleep deprivation. Thus, the total number of complete blood sets were from fourteen subjects. The high inter-individual variations in plasma and culture medium sample parameters made it clear in retrospect that more subjects would have improved the statistical analyses.

There are a number of factors that could account for the intersubject variation observed in some parameters. Diet and body composition could be major contributors to this variation; diet was standardised for all subjects on Days 1 and 2, but prior to and after this diet was at the subject's discretion. There was poor compliance with food diaries and no information on body fat mass. There are individual differences in the capacity to tolerate sleep loss; greater amounts of SWS render an individual more tolerant to sleep loss. In this study subject's normal sleep patterns were not observed before or after sleep deprivation, so accounting for greater amounts of SWS and the nature of recovery sleep could not be made. There are complex relations between the immune, endocrine and nervous systems, as well as the bi-directional relationship between cytokines and sleep. Components of each system interact, affect and are effected by one another. Alterations to the parameters measured as a consequence of sleep loss affecting components of any one of these systems should be taken into account.

It was very disappointing that the preparation of samples to monitor neutrophil function was not of sufficiently good quality for acceptable analysis. For future studies, another cell function test, such as neutrophil bactericidal ability or elastase release, lymphocyte proliferative ability, NK cell cytotoxic activity etc. should be used in addition to oxidative burst. However, to

do so would require sufficient personnel to be available to undertake the preparation and assays at the appropriate times.

Future Research

Future research should look to establishing any relationships between circadian rhythm and sleep on IL-8 and GLN, and more studies documenting cytokine activity throughout the night of sleep deprivation are needed. The significance of increased circulating leptin during sleep and whether this impacts on immune function or how it contributes to restoration should be examined. Insight into the cellular source of variations in cytokine levels during normal and disturbed sleep would be useful to determine the prevailing type of immunity in sleep and the consequences of sleep loss. Measurement of the predominant Th1 and Th2 cytokines, IL-2, IL-12, IL-4 and IL-10, after sleep loss would advance knowledge in this area. It would be interesting to measure p[FT] or the p[FT]/BCAA ratio to indicate possible central fatigue. Long term follow up of incidence of illness after one night's sleep deprivation would be constructive, as would stricter control over diet or detailed food diaries.

The cumulative effect of fatigue already experienced in excessive physical training or that induced by a negative energy balance may assist in exacerbating immunodepression due to sleep loss. Previously, Mullington et al. (2003) observed the normal nocturnal rise in circulating leptin was reduced during one night of sleep deprivation. The impact of this on immune function has not previously been considered. Given that sleep is perceived as a restorative process, then maybe there is more to the nocturnal enhancement of leptin than simply to suppress appetite during the night.

CONCLUSIONS

It was clear that the night's sleep deprivation effected changes in terms of increased sleepiness and fatigue, decreased cognitive function, vigour and physical performance. Some changes did occur in circulating cell numbers and cytokine production which were indicative of an acute phase response to an apparently perceived immune challenge the morning after losing one night's sleep. Gln supplementation had an effect on some cell numbers; its effect on cytokine production was very variable and, in the case of IL-8, unexpected. This is likely to be

due to the different type of stress involved. Gln supplementation did have a positive, beneficial effect on cognitive function in terms of mental arithmetic and logical reasoning tests.

The effect of sleep on some immune parameters has been documented, but how does sleep restore the immune system? “Restore” means to bring back, reinstate/return something to a former condition or repair [*Oxford English Dictionary*]. Further investigation into how this is achieved during sleep by circulating cells, such as NK cells, monocytes and lymphocytes, cytokine production and blood metabolites, would not only indicate the importance of sleep, but would also help understand why sleep loss results in immunodepression.

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Appendix I. Effects of Within-Subject Standardization

To perform a within-subject standardization, one uses all observations on a single subject to transform that subject's scores into subject-specific standard scores. Each subject's scores then have a mean of 0 and an sd of 1 and, nominally, they range from -3 to +3. This transform changes the relative emphases on intra- and intersubject variability within a data set, increasing the latter substantially. Specifically, it reduces the relative amount of intersubject variability, increases the relative amount of intrasubject variability and reduces the relative intergroup difference in the data set. The increased relative emphasis on intrasubject variability in repeated measures studies generally overcomes the relative reduction in intergroup differences and allows repeated-measures treatment effects to be described more clearly.

In this data set, relative intersubject variability was reduced by 29%, relative intrasubject variability was increased by a factor of 2.6, and the relative intergroup difference was reduced by 69%. The details for this data set follow.

Intersubject Variability

Within-subject standardization reduced the relative amount of intersubject variability in the data set. There were 21 observations taken on 15 subjects in two groups: 8 subjects in one group and 7 in the other. Thus, there were 42 observation means and related standard deviations (sd). The grand mean, across-subject sd (via squared sd) was 12.85 correct responses per minute (corr/min) for the sd of the 42 observations. The $(21 \times 15 =)$ 315 individual observations ranged from 12.3 to 80.3 corr/min, for a total range of 67.96 corr/min. The ratio of the grand mean sd to the range of observations is used here as a coefficient of relative variability. For the raw data, this relative coefficient was 0.189. For the standardized data, the grand mean sd was 0.757 standard deviation units (sdu) and the absolute range of individual, standardized observations was 5.63 sdu, giving a smaller relative coefficient of 0.134. Thus, the relative across-subject variability was reduced by 29% within the range of observed scores.

Intrasubject Variability

Within-subject standardization increased the relative amount of intrasubject variability in the data set. The grand mean intrasubject sd for the 15 subjects was 4.72 corr/min, giving a relative coefficient of 0.069 (using the range denominator, above). The grand mean intrasubject sd for the standard scores was, by definition, 1, giving a larger relative coefficient of 0.178. This was a relative increase of 2.6x for the amount of intrasubject variability in the data set.

Relative Difference Between Groups

Within-subject standardization reduced the relative intergroup difference in the data set. For the raw data, the mean, absolute group difference across the 21 observations was 30.89 corr/min, giving a relative intergroup difference of 0.228 (using the range denominator, above). For the standardized data, the mean, absolute group difference across the 21 observations was 0.396 sd, giving a smaller relative intergroup difference of 0.070. This was a relative reduction of 69% in the mean intergroup difference.